

METHODS OF ANALYSIS OF CERAMIDES
AND THEIR RELATION TO MILK PRODUCTS.

by

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E. George.
EDWIN ERNEST GEORGE.

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Memorandum

Photography was carried out on an overhead Polaroid camera model 3000; black and white type 107 land film pack was used.

Mass spectra were obtained on an EAI QUAD 300 quadrupole mass spectrometer. Infra red spectra were determined on a Beckman Model 33 infra red spectro-photometer in CCl_4 unless indicated. N.M.R. spectral determinations were made on a JEOL JNM-4H-100 instrument using T.M.S. as internal standard. Visible or ultra-violet spectra were determined on a Hitachi-Perkin-Elmer Model 124 Double-beam Spectrophotometer.

G.L.C. was carried out on a Shimadzu gas chromatograph Model 4 BPF with a flame ionization detector. The column packing consisted of 3% GE SE-30 on 100-120 mesh Gas-Chrom Q (silanized).

TLC was carried out on a Kieselgel G nach stahl. Melting points are uncorrected and were determined on a Gallenkamp Apparatus.

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Abstract

The analysis of milk lipids, especially ceramide, by thin layer chromatography is described. The variation of ceramide in milk and milk products under different external conditions is noted. The fatty acid composition of milk ceramide and sphingomyelin is determined by gas-liquid chromatography of the trimethylsilyl derivatives after separation by TLC followed by hydrolysis. Quantitative methods of analysis of ceramides are developed, and the ceramide in milk and milk products estimated by picrylsulphonic acid. Finally, some synthetic ceramides are prepared and analysed by TLC, infra red spectroscopy and mass spectroscopy, the methods to be used (with GLC) in the future for the complete analysis of the varied components of milk ceramides. Some anomalous TLC spots are observed, with structures possibly different from the usual straight-chain form of ceramide.

INTRODUCTION

The field of sphingolipids is comparatively new, with few papers published on particular aspects of the field. My interest is in food science and I appreciate the opportunity to investigate these compounds in one of the major groups of foodstuffs consumed by man, that is, milk and milk products. No mention is made of sphingolipids in food and health acts, although dietary ceramides could have undesirable effects on one's health. Recent findings by Dr. Polya and Dr. Parsons⁽⁴³⁾ have confirmed that (1) ceramide is a powerfully thromboplastic lipid and is a relevant factor in atherosclerosis and (2) there is a transient appearance of free ceramide on stress.

Some studies on sphingolipids in one or two milk products have been carried out so far, only with respect to sphingomyelin, present to approximately 0.06% in normal milk ($\frac{1}{3}$ of the total phospholipids), and cerebrosides in which even smaller concentrations have been found. Neither free ceramide nor gangliosides have been reported in milk or its derivatives to my knowledge. It is the aim of this thesis, and also that of future work, to develop methods of detection and analysis of free ceramide in these compounds.

Their detection is described in Chapter One by TLC, with some analytical data on their composition obtained by GLC. Chapter Two compares some quantitative methods of analysis; very few of these exist at present, and none are useful in the low micro-gram quantities ($\leq 5\mu\text{g}$). For quick, routine assays of very small quantities of free ceramide in milk, blood and tissues, this problem deserves a lot of attention in the future.

The Samuelsson and Samuelsson procedures for estimating ceramide in sphingolipids can be applied when a larger quantity of material is available; mass spectroscopy, coupled with infrared spectroscopy, proves to be a useful tool for the structural analysis of ceramides, and thus Chapter Three is devoted to some preliminary experiments on these subjects.

Synthesis of ceramides by known methods not only provide some pure ceramide standards to be used for the future analysis of milk ceramides, but also gives evidence of some suspected anomalous ceramide structures as observed by TLC and their spectra, to be studied in the future.

Chapter One

ANALYSIS OF MILK LIPIDS BY CHROMATOGRAPHY

Materials and Technique

The thin layer chromatography (TLC)^{1,2} was carried out on thick glass plates 20cm in width, and varying from 5 to 20cm in length for analytical plates, or 40cm to 1 metre in length for preparative plates. A thin film of Kieselgel G nach Stahl (non-fluorescent with about 13% calcium sulphate binder) was applied evenly to five 20cm plates at a time by means of a commercial spreader. For analytical plates, about 300 μ thick, a slurry of 30g silica gel to 57cc distilled water was used, while for preparative plates, about 1mm thick, a thicker slurry was used - 100g silica gel to 175 ml water. The layer thicknesses quoted refer to the aqueous slurry, and the thickness of the dried layer is somewhat less; it depends on the rate at which the spreader is drawn over the plates and is inversely proportional to this rate. It was found, however, that variations in the layer thickness did not normally affect to a great extent the chromatographic separations or R_f values of the sphingolipids, phospholipids, and neutral lipids used throughout the studies; if analytical plates were overloaded, there was an increase in R_f values and less effective separations.

After air drying for 30 minutes, the plates were placed in an oven set at between 80°C and 110°C for 15 to 20 minutes. The activation time and temperature were found to be critical for ceramide separations and mobility on TLC plates. Strong activation, say 1-2 hours at 110 - 130°C, was found to give ineffective separations for the four main groups of bovine brain ceramides. In fact, experiment shows that most chemically bound water must remain on the plates for efficient separations of ceramides, cerebrosides, and phospholipids.³ Reproducible R_f values for lipids in TLC were best obtained by just drying the plates at 80 - 100°C for 20 to 30 minutes. After drying

the plates were stored in a cabinet, the atmosphere of which was equilibrated with slightly hydrated silica gel desiccant.

TLC was carried out in glass chambers (Camag) the walls of which were lined with filter paper and allowed to become thoroughly wetted with the solvent 2 hours before use. Lining the tanks caused a reduction in edge effects, reduced the time for running a chromatogram by a third, and gave slightly lower but more constant R_f values since the atmosphere of the chamber was more uniformly saturated with solvent vapours. (4) Solvent to a depth of 1 cm was used to develop the plates.

The two solvent systems which were used consistently throughout this work, for both analytical and preparative separations, will be designated for future reference as solvent A and solvent B. Their compositions are as follows:-

<u>Solvent A</u>	<u>Solvent B</u>
50 chloroform	65 chloroform
50 carbon tetrachloride	25 methanol
10 methanol	4 water
0.5 water	

Solvent B was developed by Gloster and Fletcher (1966) (3) for the analysis of phospholipids of blood serum by TLC on silica gel H.

R_f Values of Some Lipids Used

Table 1.1 gives R_f values obtained for various lipids used in TLC on silica gel G using both solvents A and B. The values obtained for phospholipids are similar to those obtained by Gloster and Fletcher.

$$R_f = \frac{\text{Distance moved by substance}}{\text{Distance moved by solvent front}}$$

T A B L E 1.1

<u>Lipid used</u>	<u>Solvent A</u>	<u>Solvent B</u>
Triglycerides (from milk)	0.98	1
*Cholesterol stearate	0.91	0.99
*Cholesterol	0.87	0.93
*Ceramides (ex-bovine brain)	0.62, 0.37, 0.14, 0.07	0.98, 0.96, 0.88, 0.82
† Ceramides (ex-bovine muscle)	0.42	0.96
*Cerebrosides (ex-bovine brain)	0.13, 0.10	0.73, 0.68
*Phosphatidyl ethanolamine	0.03	0.53
† Phosphatidyl choline (L- 4 -Lecithin)	0	0.30
*Phosphatidyl inositol	0	0.24
*Sphingomyelin	0	0.19
Lysolecithin (from milk)	0	0.11

The above lipids were obtained from the following chemical companies, as indicated by the superscripts: *:- Koch-Light Laboratories, Ltd.; † :- Sigma Chemical Co.; ‡ :- Fluka A.G, Chemische Fabrik.

Koch-Light ceramides consist chromatographically of four major groups, corresponding to the four R_f values given, while Sigma ceramides give a single TLC spot only. Up to six spots have been observed for the former on a very good separation, but only one for the latter;

several solvent systems were tested, including solvent B saturated with carbon tetrachloride, but Sigma ceramides could not be further resolved by TLC.

General Reagents for the Detection of Lipids after TLC

(1) Sulphuric Acid, 50% aqueous was sprayed on the plates which were then heated in an oven at about 450°F for 20 - 30 minutes, whereupon all lipids (and any other organic compounds) were charred and appeared as grey or black spots, depending on the nature and concentration of the lipid. Potassium dichromate incorporated in the sulphuric acid to 0.6% by weight is more efficient for the charring of some lipids such as phospholipids and cerebrosides. Rouser et al^(5,6) have used this reagent for quantitative analysis of lipids by charring-transmission densitometry.

(2) Ammonium sulphate, 5% on the weight of silica gel, was incorporated into the plate coating. All lipids gave brown to black spots on a white background and longer heating time was necessary than with sulphuric acid.

(3) Iodine vapour, the plates were placed in a closed container containing an excess of iodine crystals, whereupon yellow or brown spots developed to different intensities, depending upon whether the lipid was saturated or unsaturated. Unsaturated lipids absorbed some iodine by dissolution.⁽⁷⁾ For lipids to be used for quantitative or structural analysis this reagent was not used since oxidation products form irreversibly. It was found useful, however, to detect lipids on pre-

coated Eastman Chromatogram sheets with plastic backing using the Eastman chromatogram apparatus Model 104 for TLC.

(4) Eosin (Tetrabromofluorescein), applied by spraying 0.01% aqueous onto the plate, was found very satisfactory for the detection of the following natural and synthetic lipids encountered during the course:- neutral lipids: triglycerides, cholesterol esters, cholesterol, fatty acids; phospholipids: cephalins (phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol), lecithin (phosphatidyl choline), lysolecithin; sphingolipids: ceramides, sphingosine, dihydrosphingosine, cerebroside, sphingomyelin. The lipids showed up as a bright yellow bands when in relatively high concentration on a fluorescent green background under ultraviolet light. As little as $1\mu\text{g}/\text{cm}^2$ of lipid could be detected by this non-destructive reagent.

Other fluorescent detectors were tried also: 0.01% aqueous rhodamine 6G⁽⁸⁾, gave yellow spots similar to eosin. 2',7'-Dichlorofluorescein alone did not detect sphingolipids and phospholipids, thus silica gel GF 254, which contains this indicator, was not used for TLC. However, if the thin layer of silica gel GF 254 was - on the advice of Dr. E.J. Browne - impregnated with enough 1,2,4-triazole, the screening effect observed with sphingo- and phospho-lipids due to 2',7'-dichlorofluorescein was eliminated, and the lipids became fluorescent under ultra-violet light.

Reagents for the Detection of Sphingolipids(1) Chlorine substitution followed by KI starch or benzidine

The detection of some brain cerebroside^(9, 10), sulphatides, sphingomyelins, and ceramides (N-(2-hydroxy-lignoceroyl) sphinganine and N-palmitoyl sphinganine in particular)⁽⁹⁾ has been carried out on paper and thin layer chromatograms by firstly reacting with sodium hypochlorite (chlorox) in benzene-glacial acetic acid (10:1) or simply with chlorine gas, followed by benzidine to give a blue colour. Also tert-butyl hypochlorite has been used as the chlorinating agent,^(11, 12) followed by KI-starch, for the detection of peptides, nucleotides and other N-H containing compounds on paper chromatograms. Sphingolipids were not tested by this reagent, but the method was tried in this project and proved successful with ceramides, cerebroside, sphingomyelins, sphingosine and dihydrosphingosine: very low concentrations of these sphingolipids could be detected - less than $1 \mu\text{g}/\text{cm}^2$. The method of detection was as follows: t-butyl hypochlorite was prepared⁽¹³⁾ (45ml, 80% yld), and sprayed as a 2% solution in cyclohexane onto a silica gel thin layer plate containing spots of sphingolipids. The plate was dried in a fume cupboard for 30 minutes under a direct blast of cold air from a hair-dryer, and then sprayed with an aqueous solution of potassium iodide (1%) - soluble starch (1%). The lipids were visualised as blue-black spots on a white background. After 10 minutes however, the background began to become light blue due to atmospheric oxidation.

A variation of the method consisted in chlorination of the amide by exposing the TLC plate to dry chlorine gas in a closed container for an hour, and then spraying with potassium iodide-starch solution.⁽¹⁰⁾ This method was found less sensitive ($\geq 5 \mu\text{g}$), and gave rise to some background colour.

(2) Malachite green:

The detection of a great variety of lipid materials has previously been reported⁽¹⁴⁾ by the use of the cationic stain, malachite green. The dye was also found to identify ceramides and cerebroside, and all other milk lipids by comparison with charring on TLC plates. The lipids were spotted on silica gel G plates and chromatographed in solvent A. The chromatograms were then uniformly sprayed with an 0.5% aqueous solution of malachite green to produce a deeply blue-coloured surface. The plates were air-dried 30 minutes and sprayed lightly with 2% sodium metabisulphite in 2N HCl to reduce all unbound stain to an orange colour, leaving the lipids as blue spots. This method would be unsuited to quantitation however with the reducing reagent used, as the plates immediately began to partially reoxidize. For qualitative purposes the method was found to be suitable for sphingolipids and cholesterol to greater than $5\mu\text{g}/\text{cm}^2$.

(3) Metal complexing:

The effect of metal ions on some sphingosine bases has previously been tested⁽¹⁵⁾ with no evidence of complex formation, but the ability of ceramides to form complexes on TLC plates with some transition metal ions remained to be studied. Ceramide was spotted on the plate, followed by some aqueous solution of a metal salt, and chromatographed in solvent A. The acetates and chlorides of Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Hg^{2+} were tried, with no success. Detection reagents used were potassium ferrocyanide (Fe, Cu); dimethylglyoxime (Ni); 8-hydroxyquinoline (Co); H_2S (Hg). The metal salts remained at the origin, and subsequent treatment with I_2 vapour showed that the ceramide was not retarded at all by the metals.

(4) Phosphomolybdic acid:

Phosphomolybdic acid has previously been used to detect phospho-

lipids, followed by staining for choline with ninydrin⁽¹⁶⁾, as well as quantitative analysis of phospholipids, cholesterol, cholesterol esters, free fatty acids and triglycerides in human serum lipids⁽¹⁷⁾.

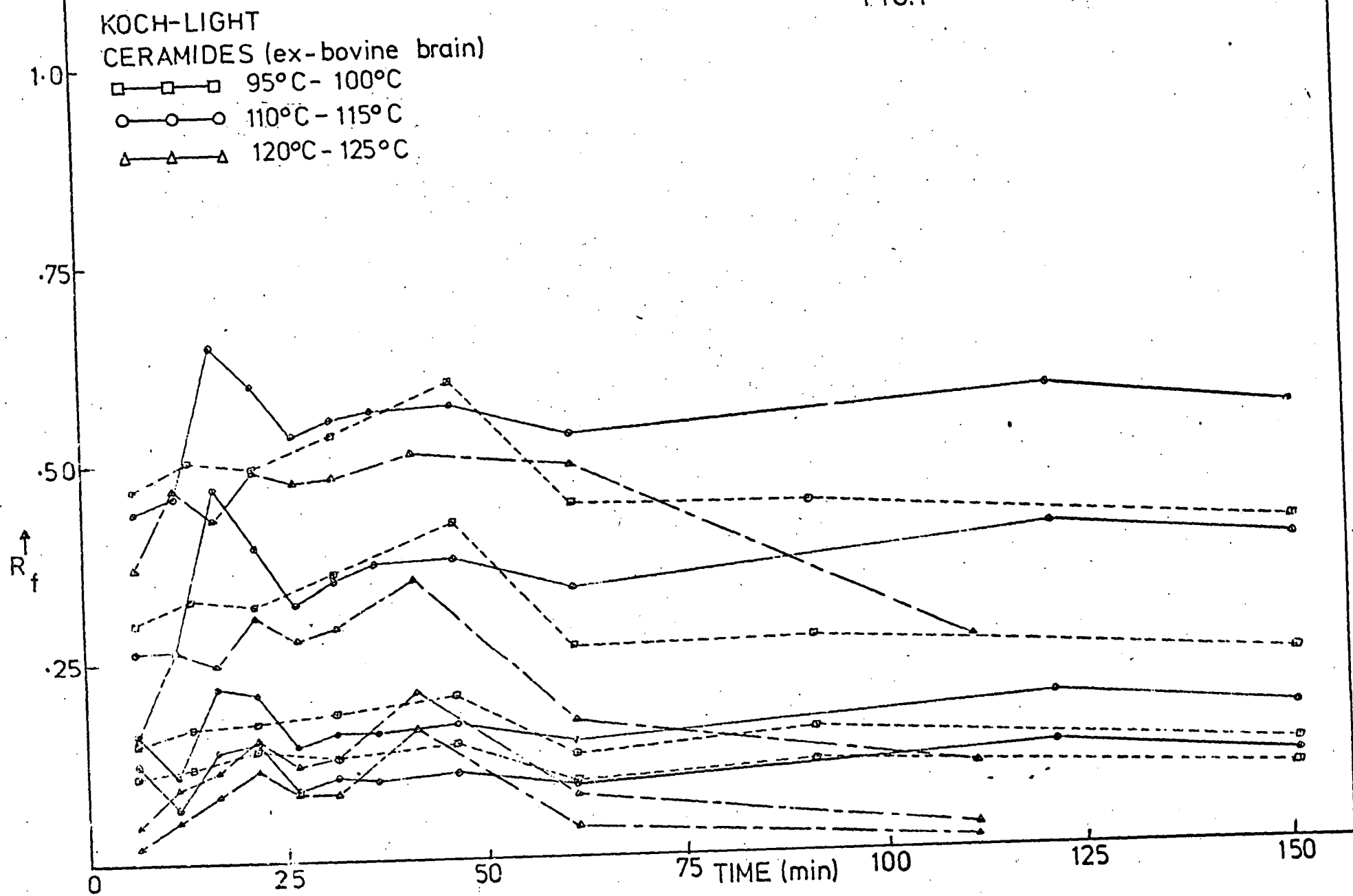
Ceramides, cerebrosides, and sphingomyelin were subjected to TLC in three different concentrations (0.5, 2, 10 μ g), and the plates sprayed with a 10% methanolic solution of phosphomolybdic acid. Upon heating 1-2 minutes at 110°C, blue spots of reduced molybdate on a light yellow background were observed, formed due to the unsaturation of the sphingolipids. A concentration of 0.2 μ g/cm² of lipid was just detectable by this reagent, and it was used to provide a means of quantitation by spectrodensitometry (see Ch.2.).

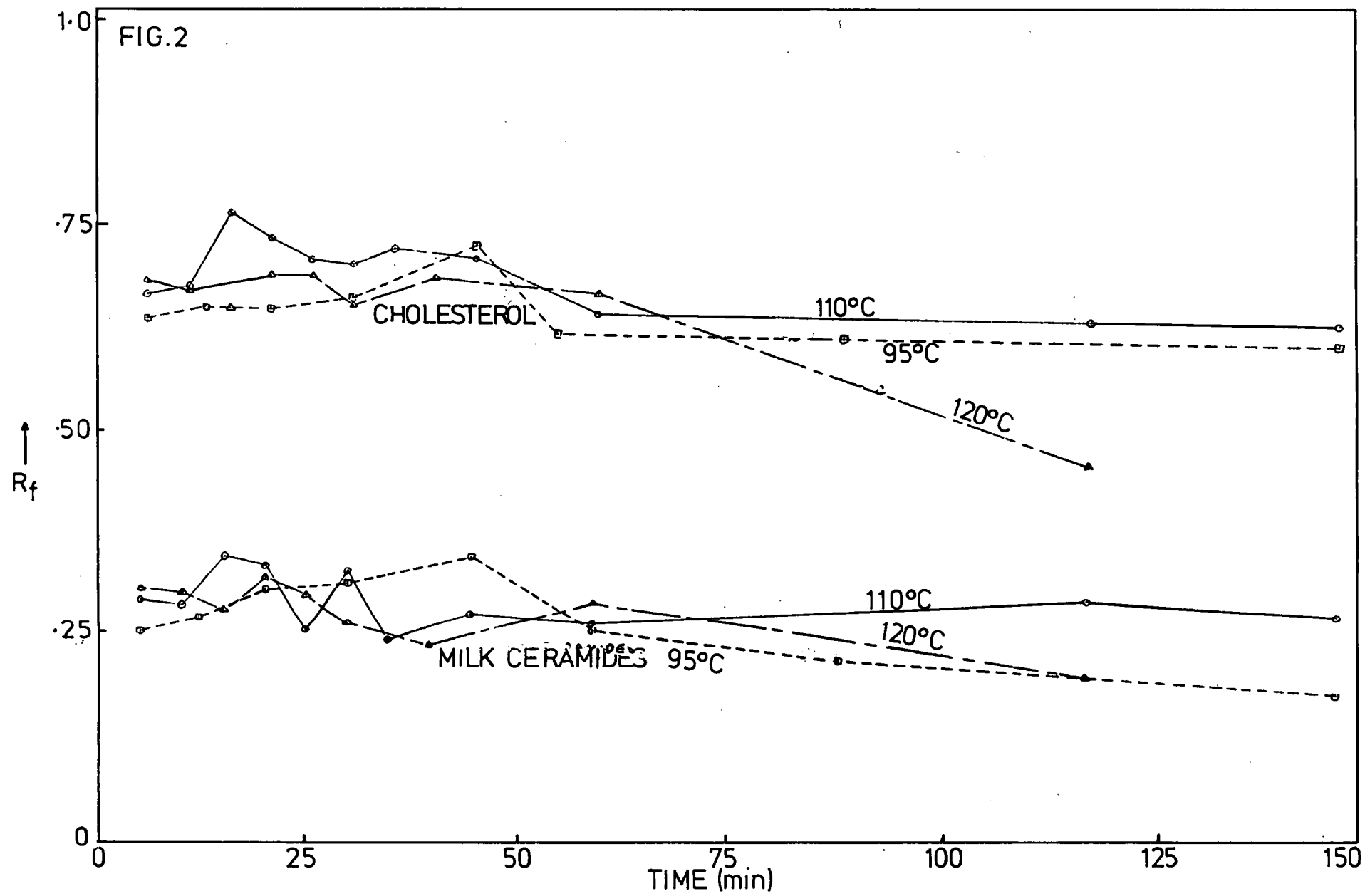
Effect of Temperature and Time of Activation on R_f Values of Ceramides, Cerebrosides, and Cholesterol

For studies of time variation at constant temperature, an oven (accurate to $\pm 2^\circ\text{C}$) was kept at a predetermined temperature. TLC analytical plates (20cm x 10cm) were prepared, and air-dried for 30 minutes before heating. The plates were removed from the oven as quickly as possible as time progressed and cooled in a cabinet containing dessicant for 30 minutes. They were then spotted with the lipid solutions immediately, placed in TLC tanks containing solvent A, and the solvent allowed to rise to a predetermined solvent front. Results for temperatures of activation between 80°C and 130°C are illustrated by figs. 1 and 2.

The greatest changes in R_f values are observed within the first hour, particularly the first half-hour. The optimum conditions for Koch Light ceramides, as well as other lipids used, seems to be 15-20 minutes at 110°C. For the most reproducible conditions, however, a drying time of 30 minutes at 95 - 100°C or 100 - 105°C could be used, where the plates would only be slightly activated.

FIG.1





TLC of Lipids From Milk Products

The following types of milk products were examined for lipids, ceramides in particular, by TLC (figures in parentheses indicate percentage butterfat⁽¹⁸⁾): "Bakers" pasteurised full-cream milk (3.6), "Meadowlea" butter (83), "Carnation" full-cream evaporated milk (8.3), "Nestles" full-cream powdered milk (27), cream (30), skim milk (0.2), human milk (4.0 - 3 days after birth). As shown by the photographs of the TLC plates displaying the milk products (plates 1-4), the quantity of ceramide relative to the butterfat content can be approximately assessed.

The lipids were extracted from the milk samples by the method of Gloster and Fletcher⁽³⁾ as follows: 2 mls of the milk product were shaken with 40ml of chloroform-methanol (2:1) in a glass-stoppered flask, and stood at room temperature for 30 minutes with frequent shaking. The mixture was then shaken with 8 ml of 0.1 M sodium chloride solution and the lower chloroform phase filtered using phase filter-paper (Whatman 1-PS). The chloroform-methanol phase was evaporated to dryness under a stream of nitrogen or by a rotavapor and the liquids redissolved in 2 ml of chloroform; 10 μ l of this solution were used for spotting.

Plates 1 and 2 illustrate the lipids present in normal pasteurised milk, evaporated milk and full-cream powdered milk, using solvents A and B respectively. Free ceramides are observed to be present on plate 1 as a major band around R_f 0.45, and a minor band around R_f 0.17. Triglycerides are present as the large majority of lipids as stands out by the intense black spots at the solvent front. Cholesterol is present to a greater extent than ceramides (R_f 0.85 - 0.9), and a proportional amount exists in all milk products relative to the butterfat. Plate 2 shows clearly the presence of the phospholipids lysolecithin (R_f 0.1),

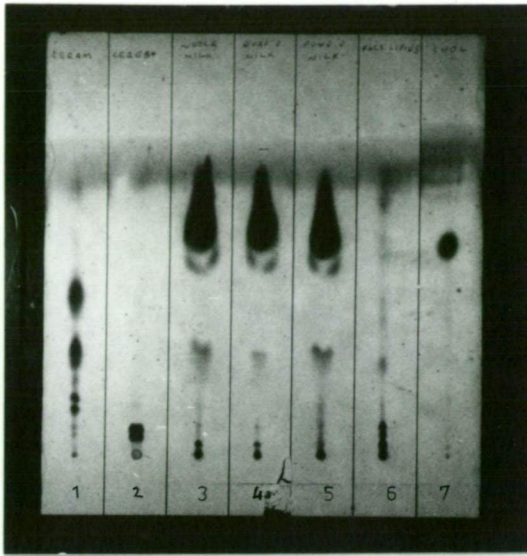
Sphingomyelins (R_f 0.2), lecithins (R_f 0.3) and cephalins (R_f 0.5). Cerebrosides are also known to be present in milk and milk products, though very faint here (R_f 0.7) Morrison^{19,20} has analysed by gas-liquid chromatography and mass spectrometry the long-chain bases and fatty acids of sphingomyelin, glucosylceramide and lactosylceramide in spray-dried buttermilk powder.

Plate 3 shows the variation of ceramide in skim milk and cream, samples of which were obtained by centrifuging whole milk (pasteurized) in the laboratory. The cream was removed from the top fraction, and the lower fraction used for the skim milk. 1% of the extracted milk lipids from 2 ml of the milk samples were used for TLC analysis.

The majority of the ceramide is observed to be present in the skim milk, judging from the intensity of the charred lipid around R_f 0.4 for each sample. Some of the less mobile ceramide is observed also to be present in skim milk, whereas none can be seen in the cream extract for that concentration. This may be expected, since ceramides contain two or more hydroxy groups, and an amide linkage; hydrogen bonding and electrostatic attraction would tend to keep the ceramide in the aqueous phase associated with the milk proteins.

The R_f value for the major band of milk ceramides, the same for all milk products, is observed at R_f 0.45, corresponding to a little higher than the position of Koch-Light ceramides band 2. The latter are rich in hydroxy fatty acids⁽²¹⁾, and thus the reason for their retention on TLC plates. However, milk ceramides have only a small proportion of hydroxy fatty-acids, and a large proportion of unsaturated fatty acids, as shown later by GLC analysis.

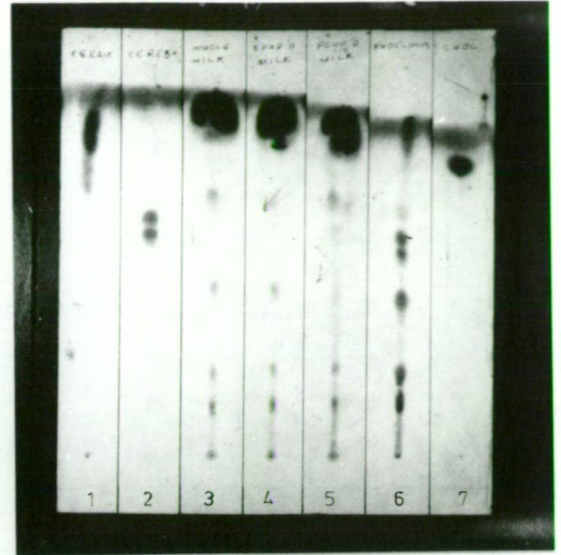
Plate 4 compares the lipids in fresh cows' milk with an equal quantity from human milk from a mother 3 days after birth. The ceramide content is observed to be much larger in human milk.



1

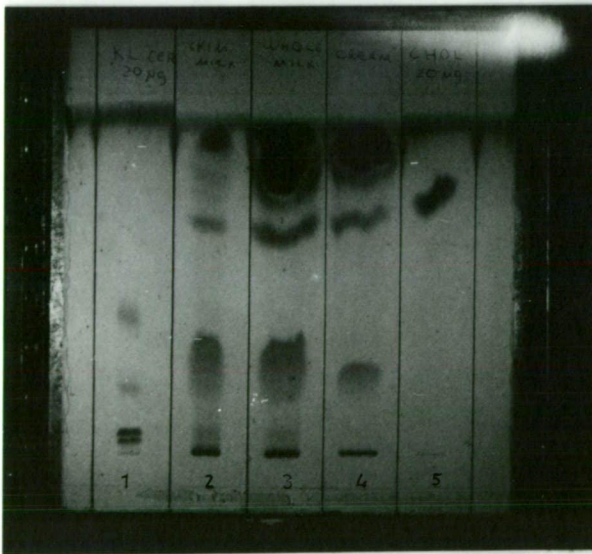
(1) Brain ceramides; (2) Brain cerebroside; (3) Whole milk (pasteurised) lipids; (4) Evaporated milk lipids; (5) Full-cream powdered milk lipids; (6) sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine, cerebroside; (7) cholesterol

Plate 1. - sol. A



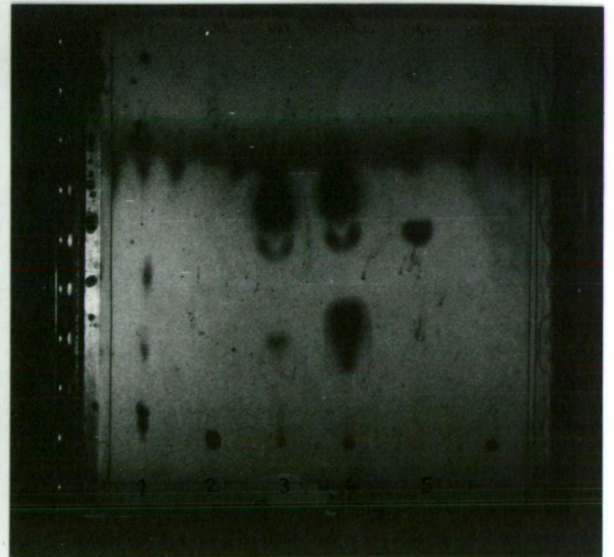
2

Plate 2. - sol. B



3

(1) Brain ceramides
(2) Skim milk lipids
(3) Whole milk lipids
(4) Cream lipids
(5) Cholesterol



4

(1) Brain ceramides
(2) Brain cerebroside
(3) Cows' milk lipids
(4) Human milk lipids
(5) Cholesterol

Comparison of Solvent Power of Acetone, Isopropanol for Milk Ceramides and Cholesterol

Human milk was washed twice with acetone or isopropanol and their solvent powers compared by TLC of the extracts of the residues and washings; results are shown on plates 5 and 6.

Ceramide is observed to be present in relatively large concentration corresponding to the second Koch Light ceramide spot at about R_f 0.5. The chromatograph shows that practically all the ceramide is extracted by both solvents as well as all the cholesterol, other sterols and triglycerides. The solvent power of isopropanol is noted to be greater than that of acetone for cerebrosides (R_f 0.08); practically all cerebrosides are extracted by one wash with isopropanol.

Experiments were carried out to observe the effect of extracting whole cows milk with increasing proportions of acetone and isopropanol. The preparations were, for milk:solvent, 1:1, 1:2, 1:4, 1:8, 1:16. The coagulated protein and remaining lipids were centrifuged, the top layers drawn off, and the solvent evaporated to dryness under a stream of nitrogen. The lipids were then extracted from both the residues and extracts by the Gloster-Fletcher method, the solvent evaporated to dryness under nitrogen and lipids taken up in 1 ml CHCl_3 ; 10 μl were used for spotting on TLC plates. Results are shown on plates 7 and 8.

It is observed that isopropanol has the greater solvent power for both triglycerides and cholesterol; practically all the cholesterol is removed by the system 1 milk:8 solvent or more. The ceramide concentration seems to be only slightly affected on increasing the solvent proportion. Approximately equal amounts appear in both the residues and extracts.

Quantitative analysis was carried out for cholesterol and ceramide on each of the fractions in the residues and extracts by

the following method. The total lipid was applied as a chloroform solution to 20 cm x 20 cm preparative plates and chromatographed in solvent A. The sides of the plates were sprayed with 1% aqueous eosin, viewed under the UV light, and the ceramide (R_f 0.4) and cholesterol (R_f 0.8) bands marked. These were scraped off the plates, extracted twice with chloroform-methanol (2:1), the solvent evaporated under a stream of nitrogen and the ceramide and cholesterol estimated quantitatively. The methods used, described in section II, have been developed previously. (22,23)

Results for cholesterol and ceramide in 1 ml samples of milk after extraction with acetone or isopropanol are shown in table 1.2, and graphically by figs. 1.1 and 1.2

TABLE 1.2

A = acetone

I = isopropanol

e.g. A_4 = 1 milk:4 acetone

Extraction System	Cholesterol				Ceramide	
	Extract		Residue		Extract	Residue
	Abs.	1/A	Abs.	1/A	Abs.	Abs.
A_1	.05	20.0	.50	2.0	.08	.11
A_2	.21	4.8	.26	3.85	.10	.11
A_4	.36	2.8	.12	8.35	.12	.09
A_8	.43	2.33	.07	14.3	.10	.08
A_{16}	.48	2.10	.04	25.0	.11	.12
I_1	.12	8.35	.44	2.3	.10	.06
I_2	.30	3.33	.20	5.0	.13	.08
I_4	.45	2.22	.09	11.0	.12	.07
I_8	.47	2.13	.04	25	.14	.09
I_{16}	.49	2.04	.02	50	.13	.08

A plots of milk : solvent ratio vs absorbance (fig. 1.1) shows that isopropanol has the greatest solvent power for cholesterol, and slightly greater only for ceramide. As shown up by plates 7 and 8 also, it is noted that practically all the cholesterol is extracted for the milk : solvent ratio 1:8, and ceramide is equally distributed between residue and extract.

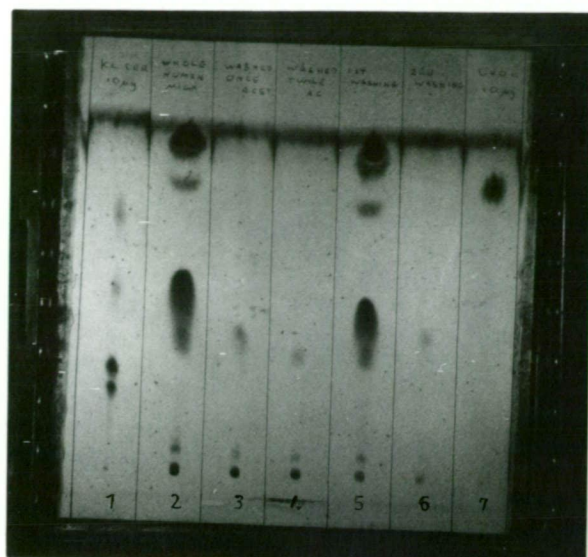
For the cholesterol remaining in the residues, the milk : solvent ratio is inversely proportional to the cholesterol concentration (fig. 1.2).

Finally, the solubility of ceramide, cholesterol and synthetic cephalin* were determined as follows. Solvent was added in 0.1 ml aliquots to an appropriate amount of lipid, warmed to completely dissolve, then stood in an ice bath whereby a turbid solution was obtained (or crystalline particles for cholesterol). The temperature was gradually raised until the compound just dissolved, at which point the temperature was noted. The following solubilities were obtained

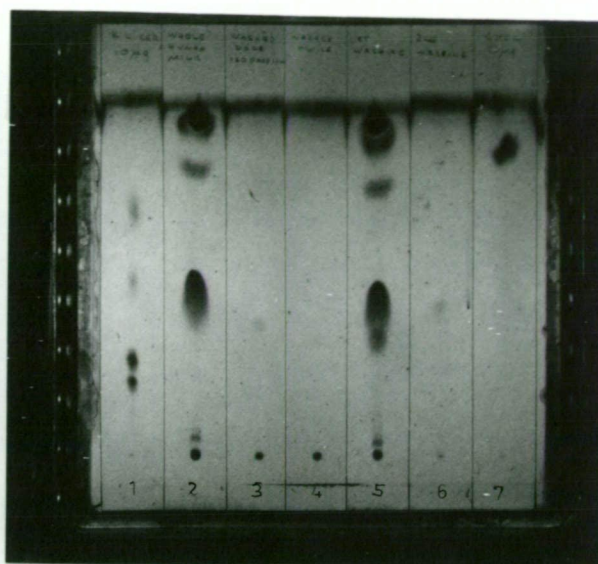
<u>Compound</u>	<u>Solubility, acetone</u>	<u>Solubility, isopropanol</u>
Cholesterol	28 mg/ml @ 30°C	100 mg/ml @ 28°C
	20 " 20°C	80 " 23°C
ceramide	0.8 " 30°C	14 " 22-28°C
	0.6 " 24°C	
cephalins	practically insoluble	< 0.2 mg/ml

Isopropanol thus has far greater solvent power for ceramide (20 x) and dissolves 4 times as much cholesterol.

*The synthetic cephalin is DL-3-phosphatidyl ethanolamine whereas the crude cephalin obtainable (Fluka AG) may be shown by TLC to contain sphingomyelin, cerebrosides, lecithin, cholesterol and a little ceramide as well as the phospholipids comprising cephalins. Washing the crude cephalin with acetone removes most of the neutral lipids, leaving the phospholipids and cerebrosides.

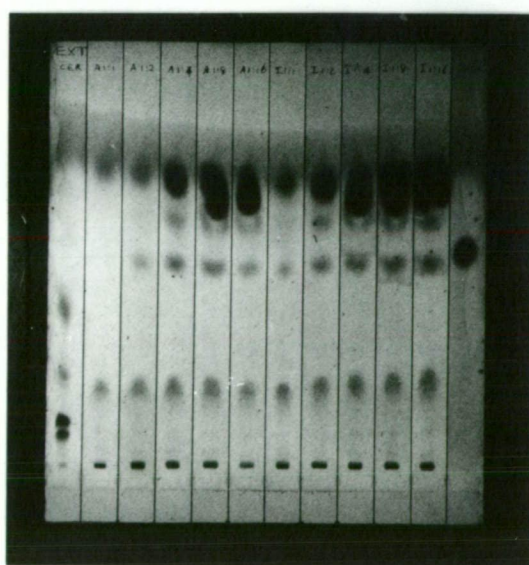


5

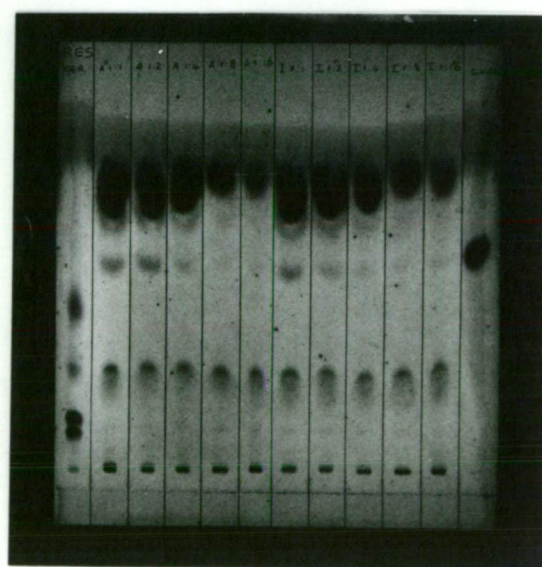


6

- (1) Brain ceramides; (2) Human milk lipids (1% from 1 ml);
 (3) Lipids, washed once) with 4 ml acetone (p.5)
 (4) Lipids, washed twice) with 4 ml isopropanol (p.6)
 (5) 1st washing; (6) 2nd washing; (7) Cholesterol.



7



8

Extracts

Residues

Plates 7 and 8 represent whole cows' milk extracted with acetone (A) or isopropanol (I). Columns are marked (e.g. in column 3, A1:2 means 1 ml of milk was extracted with 2 mls of acetone).

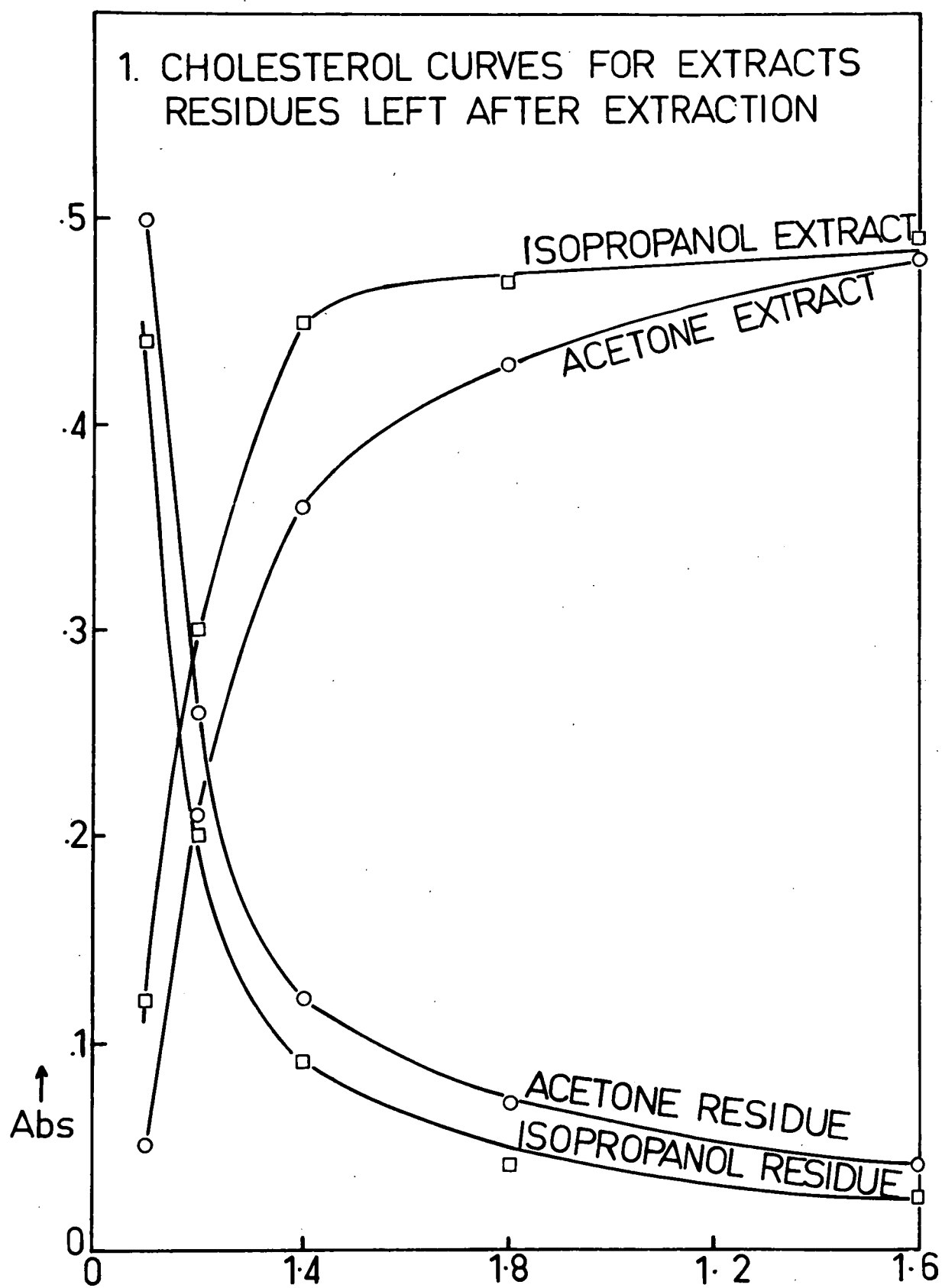


FIG. 11

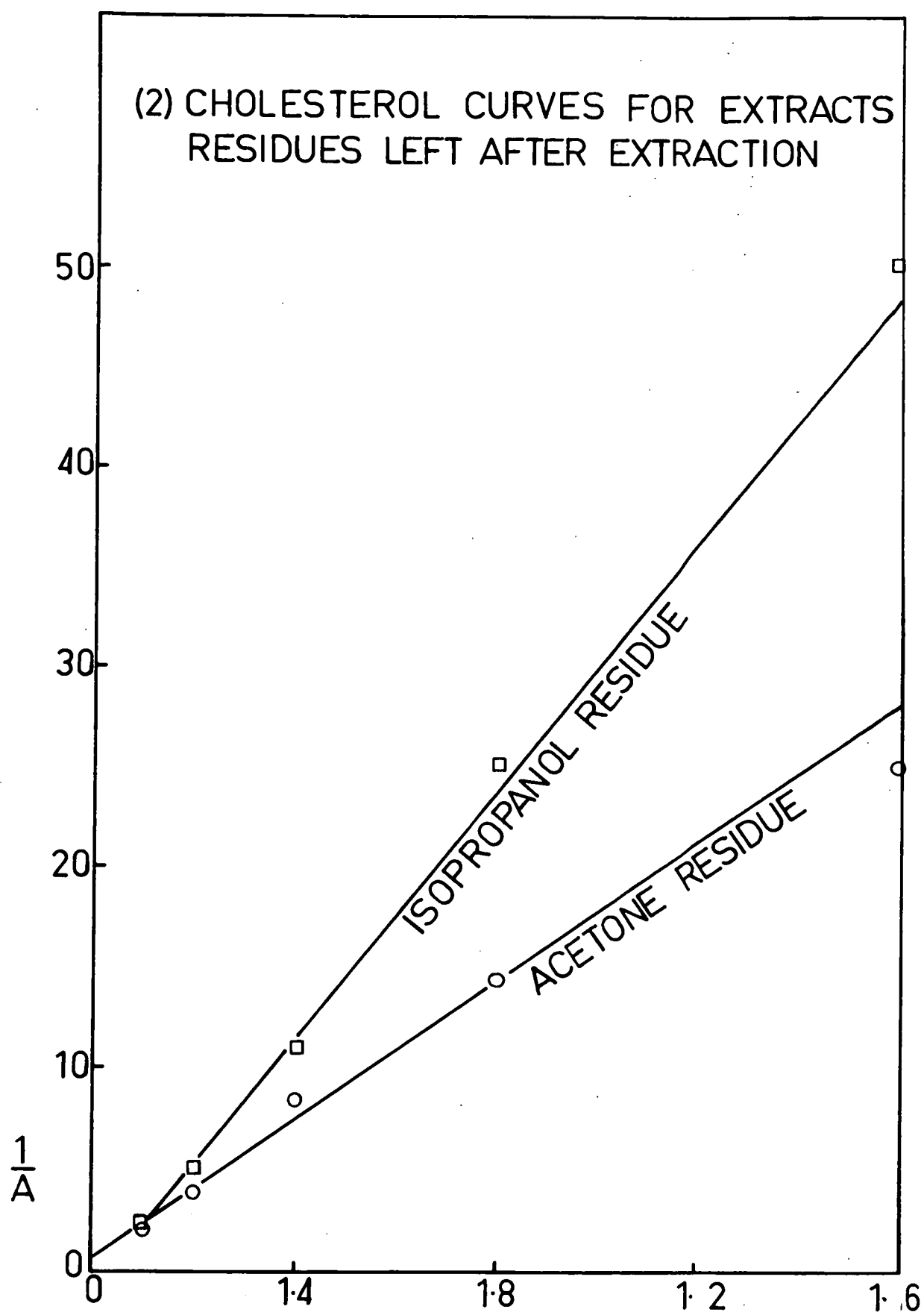


FIG. 1.2

The Effect of Different Conditions on Fresh Cows' Milk

Fresh, untreated cows' milk was subjected to varying degrees of temperature from refrigeration (4°C) to incubation (38°C). The lipids were then extracted from 2 ml aliquots at intervals by the Gloster-Fletcher⁽³⁾ method, the solution evaporated to dryness and residual lipids taken up in 2 ml chloroform. These solutions were spotted on TLC plates, the results of which are shown on plates 13 and 14.

No noticeable change compared to fresh milk is observed on refrigeration, freezing 3 days, or standing at room temperature before souring. Incubation (38°C), however, caused the milk to sour quickly, and a spot just above the major ceramide band of milk was observed in each case (pronounced in column 5, plate 13). A faint spot was also produced by prolonged boiling of milk. The spot was shown to originate from milk sphingomyelin by the following experiment. Sphingomyelin (2 mg) was dispersed into 2 ml of fresh pasteurised milk, and incubated. The lipids were extracted from 0.5 ml portions of the milk after 1 hour, 7 hours, 3 days, and equal amounts subjected to TLC. Milk was also treated with cerebrosides simultaneously, and results are shown chromatographically on plates 15, 16 and 17.

The breakdown of sphingomyelin to ceramide (+ phosphoryl-choline) is clearly seen to increase as the milk ages under incubation (38°C), by the increasing intensity of the newly formed ceramide band around R_f 0.45; this indicates the presence of the enzyme sphingomyelinase (phospholipase C) in milk. The fatty acid and long-chain base compositions of the newly formed ceramide (described later) were found to be identical to those of milk sphingomyelin.

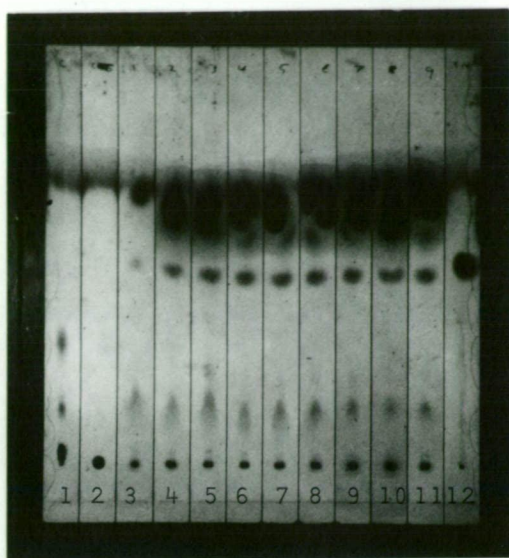
Plates 13 and 14 represent fresh, untreated cows' milk, tested at the dairy, to the conditions indicated.

PLATE 13

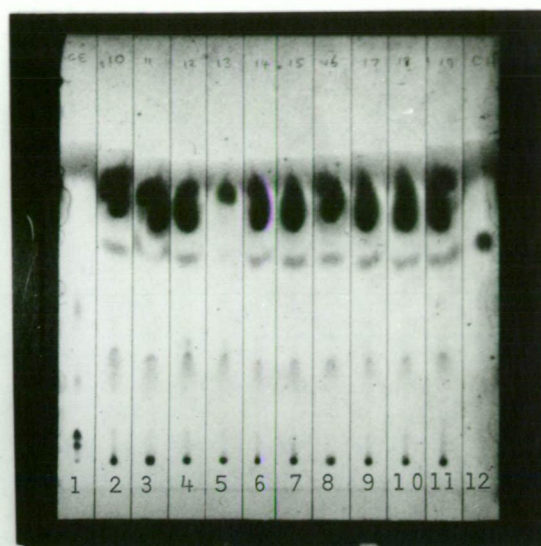
(1) Brain ceramide; (2) cerebrosides; (3) Skim milk, machine separated and tested immediately; (4) Cream from (3); (5) Incubated milk, 48 hours @ 38°C (sour); (6) Boiled 5 min.; (7) Boiled 1 hour; (8) glucose added, incubated 1 hour; (9) galactose added, incubated 1 hour; (10) frozen 3 days; (11) Whole milk, tested immediately; (12) Cholesterol.

PLATE 14

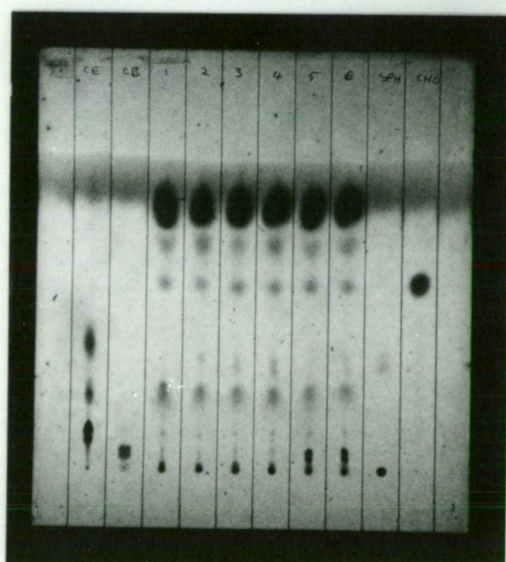
(1) Ceramides; (2) Whole milk, tested immediately; (3) Incubated 9 hours; (4) Incubated 48 hours; (5) Incubated 80 hours (whey taken without mixing); (6), (7) and (8) Left at room temperature (18°C) for 9 hours, 48 hours and 80 hours, respectively; (9), (10) and (11) Refrigerated (4°C) for 9 hours, 48 hours and 80 hours, respectively; (12) Cholesterol.



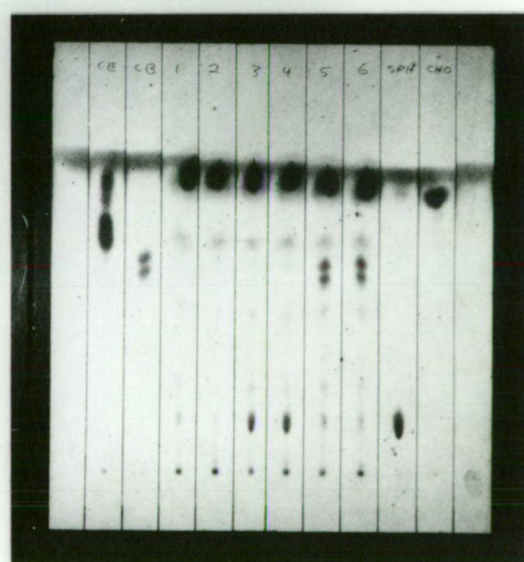
13



14



15



16

Columns are marked as follows: (CE) Ceramide; (CB) Cerebroside;
 (1), (2) Pasteurised milk incubated (38°C) 1 hour, 7 hour respectively;
 (3), (4) As for (1), (2), with sphingomyelin added (1 mg./ml.);
 (5), (6) As for (1), (2), with cerebrosides added (1 mg./ml.);
 (SPH) Sphingomyelin; (CHO) Cholesterol.

Plate 15 - sol. A.

Plate 16 - sol. B.

Separation of Milk Ceramides by Silica Gel G Column Chromatography

The use of column chromatography on silica gel G has been carried out on human serum. Complete separation of various lipid classes and lipoprotein fractions have been obtained⁽⁴⁰⁾ using silica gel G, the more conventional column packings such as silicic acid or Florisil were less effective.

A column of silica gel G 16 cm in height by $2\frac{1}{4}$ cm in width was prepared by allowing a 2:1 slurry in chloroform to settle in the column on top of a cottonwool plug. Total lipids were extracted from 1 l of fresh cow's milk (4.5% butterfat) by the following method.

Acetone ($\frac{2}{3}$ l) was added to the milk, the precipitated protein with some complexed lipid filtered off and allowed to dry under nitrogen in a large desiccator. The filtrate (ca. $\frac{1}{2}$ l) was flash distilled at 60°C under a vacuum by means of a rotary evaporator. The residue from the filtrate plus the protein was now extracted for total lipids with 1 l of chloroform-methanol (2:1) in a blender. The protein and carbohydrate were filtered off, washed once with 200 ml of same solvent, and the total combined filtrate evaporated to dryness on a rotavapor. Practically all the solvent was recovered. The extracted lipid was dissolved in 100 ml of chloroform, and added to the top of the silica gel column. Neutral lipids were eluted with 50 ml portions of chloroform, 250 ml being required to remove all glycerides, steroids and pigments. TLC on 20 cm and microslide plates as the chromatography progressed was used to indicate when all neutral lipids had passed through. The ceramides and some of the phospholipids were then eluted with 200 ml of $\text{CHCl}_3:\text{CH}_3\text{OH}(2:1)$, the remaining phospholipids being eluted with methanol. The main fraction containing the ceramides was observed to be a narrow yellow band about 3 mm thick which moved from

the top down the column at a constant rate with the eluting solvent. The combined ceramide-rich fractions were filtered to remove small silica gel particles, and evaporated completely to dryness under nitrogen in a tared flask on the rotavapor. A yellow residue of 200 mg was obtained. This residue was dissolved in a 5 ml chloroform, applied to a preparative TLC plate 1 metre long and chromatographed in solvent A. Spraying the edges of the plate with 0.1% aqueous eosin and viewing under the U.V. light revealed the presence of two ceramide bands close together at R_f 0.40 and 0.45; a very minor band was also observed between the two main bands, but was too small to be recoverable. The two main ceramide bands were individually scraped off the plate (excluding the eosin-sprayed sections), extracted with warm chloroform-methanol (2:1), and rechromatographed on two 40 cm preparative TLC plates in solvent A. The purified ceramides were recovered to yield 1.5 mg of ceramide A (R_f 0.46), and 17.0 mg of ceramide B (R_f 0.40 - the major ceramide band observed for milk products). The yield of ceramide B obtained was 65% of the theoretical yield (26 mg/l of milk by analysis).

Sphingomyelin was also recovered from the column fractions rich in this lipid, by preparative TLC twice in solvent B. One band only was obtained, at R_f 0.20 corresponding exactly to the R_f value of ex-bovine brain sphingomyelin. The sphingomyelin fraction, and both ceramide fractions, all shown to be homogeneous by analytical TLC, were stored in chloroform under nitrogen for further analysis by gas-liquid chromatography.

Ceramides were separated also from the extracted lipids of 50 g of full-cream powdered milk on a column of silica gel G 10 cm x 2 cm. A fraction collector was used (10 ml fractions) and Table 1.3 represents the compositions of the lipids obtained of the fractions by eluting solvents of increasing polarity. The fractions were monitored by TLC.

TABLE 1.2

Groupings of Chromatographic Fractions

No.	Fraction	Eluant	Composition
1	0 - 10	CHCl_3	triglycerides, hydrocarbons
2	10 - 13	CHCl_3	cholesterol, cholesterol esters
3	14 - 24	$\text{CHCl}_3/\text{CH}_3\text{OH}$ 19:1	ceramides
4	21 - 24	$\text{CHCl}_3/\text{CH}_3\text{OH}$ 19:1	cerebrosides*
5	25 - 40	$\text{CHCl}_3/\text{CH}_3\text{OH}$ 9:1	phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin
6	40 - 60	CH_3OH	Lysolecithin

*Cerebrosides were detected by anthrone⁽²²⁾ (9,10-dihydroxy-9-oxo-anthracene) which reacts with the carbohydrate portion of the molecule.

The ceramide fractions were pooled, purified by preparative TLC and stored under nitrogen for further analysis (ceramide C).

The Fatty Acid Composition of Milk Ceramide and Sphingomyelin

Beef-brain sphingolipids have been analysed for their fatty acid composition by gas-liquid chromatography (GLC) of their methyl and acetoxymethyl esters⁽⁴¹⁾. The non-hydroxy fatty acid composition was found to be similar in sphingomyelin, ceramide, cerebroside, and cerebroside sulphate, with lignoceric, nervonic, and stearic acids predominating. Hydroxy fatty acids were found mainly in cerebroside and ceramides, with 2-hydroxylignoceric, 2-hydroxynervonic and 2-hydroxystearic acids predominating. Similar fatty acid compositions of beef brain sphingolipids (supplied by Koch-Light Laboratories Ltd), have been obtained by Mejglo⁽²¹⁾, by analysis of the trimethylsilyl (TMS) derivatives of the acids; 2-hydroxybehenic and 2-hydroxyarachidic acids were also found to be present to a relatively high extent in sphingomyelin and cerebroside, and to a lesser extent in ceramides.

The pure ceramide and sphingomyelin fractions obtained in the previous section were prepared for GLC analysis by the following procedure. The lipid was hydrolysed by the method of Kates⁽⁴²⁾, by refluxing about 1-2 mg in 4 ml of methanolic hydrogen chloride (2.5%, ca. 0.7N, made by passing dry HCl gas into reagent grade methanol) on a hot water bath for 4 hours; 1 ml of water was added, and the fatty acid methyl esters extracted with petroleum ether (40-60°) quantitatively. The combined extracts were absolutely dried by anhydrous magnesium sulphate, and evaporated to dryness under nitrogen. The hydroxy fatty acids present in the residue were converted to the trimethylsilyl ethers by the addition of 100 µl of bis-trimethylsilyltrifluoroacetamide (BSTFA) followed by 50 µl of trimethylchlorosilane (TMCS), and the reaction mixture left to stand overnight in a tube with teflon-lined screw cap. About 20 µl were used directly for GLC analysis.

For long chain base recovery, the methanolic phase was concentrated

by evaporation at 40°C in a stream of nitrogen to a small volume, made alkaline with 0.5 ml of 7N sodium hydroxide, and the bases extracted with ethyl ether or chloroform. The combined extracts were dried (anhydrous magnesium sulphate), evaporated to dryness under nitrogen, and converted to the TMS derivatives as above in preparation for GLC analysis.

Gas-liquid chromatographic separations were carried out on a Shimadzu GC-4BMPF equipped with a flame ionization detector. The columns were 2m. by 3 mm. coiled glass tubes; the column packings were 3% GE SE 30 on 100-120 mesh silanized Gas Chrom Q (Applied Science Laboratories). The injection heater was at 280° and detector temperature also 280°C. Temperature programming was from 160°C to 350°C at 5°/ minute, and chart speed 5 mm / minute.

Table 1.4 gives the relative percentage fatty acid compositions of the ceramides and sphingomyelins isolated from milk previously. Retention times for the standard fatty acid methyl esters and BSTFA derivatives had been previously obtained⁽²¹⁾, and quantitative analysis of each fatty acid was done by measuring the peak area relative to the total area under all the peaks (peak area = height of peak x width at half peak height.)

A comparison of the data given in column 2 and 3 gives conclusive evidence that ceramide A, the minor component observed by TLC when milk is incubated, boiled or allowed to go sour, is derived from milk sphingomyelin (see photo 13, p. 24). The gas-liquid chromatograms of the fatty acid methyl esters and their TMS derivatives were practically identical; Fig. 1.5 shows the chromatogram for ceramide A of sphingomyelin. Fig. 1.6 shows the chromatogram obtained for the separation of the long chain bases (LCB) of sphingomyelin which was also identical to that obtained for the LCB of ceramide A.

TABLE 1.4

Relative % of fatty acids isolated from milk ceramide and sphingomyelin

Fatty Acid	Ceramide B	Ceramide A	Sphingomyelin	Ceramide C
12:0, lauric	2.0	0.3	0.2	0.7
14:0, myristic	10.3	2.3	1.7	3.0
15:0, pentadecanoic	1.4	0.3	0.4	0.3
16:0, palmitic	40.0	17.7	19.0	10.0
17:0, heptadecanoic	0.7	0.2	0.5	0.2
18:1, oleic	17.7	8.7	11.4	10.1
18:2, linoleic	22.8	19.7	14.1	70.0
18 h:0, 2-hydroxystearic	0.2	1.4	0.6	0.3
19:0, nonadecanoic	0.2	0.2	0.5	0.6
20:0, arachidic	0.1	0.2	0.3	-
20:1, 9-eicosanoic	0.1	0.2	0.4	0.3
20 h:0, 2-hydroxyarachidic	0.1	0.3	0.4	0.4
21:0, heneicosanoic	0.1	0.2	0.1	0.5
22:0, behenic	0.6	9.5	12.0	0.3
22h:0, 2-hydroxybehenic	0.1	11.7	10.8	0.4
22:6, docosahexanoic	-	0.3	0.4	-
23:0, tricosanoic	0.1	18.0	19.1	0.1
23h:0, 2-hydroxytricosanoic	-	1.0	1.2	-
24:0, lignoceric	0.1	0.4	0.6	0.1
24h:0, 2-hydroxylignoceric	0.3	0.7	0.4	0.3
24:1, nervonic	0.1	-	0.3	-
25:0, pentacosanoic	0.2	0.1	0.1	0.2
26:0, cerotic	0.2	0.1	0.1	0.1
26h:0, 2-hydroxycerotic	0.1	1.2	0.6	0.2

The predominant fatty acids found in milk sphingomyelin were palmitic, linoleic, oleic, behenic, 2-hydroxybehenic and tricosanoic acids. However, the unsaturated acids, linoleic and oleic were found to be present to only a minor extent in sphingomyelin isolated from spray-dried buttermilk powder (Morrison, ^(19,20)); in addition, lignoceric acid and the 2-hydroxy derivatives of lignoceric, tricosanoic and palmitic acid were present to a large extent as shown by mass spectra on their trimethylsilyl ethers after GLC.

The fatty acid composition of milk ceramides (from fresh milk-ceramide B and full-cream powdered milk - ceramide C) is given in columns 1 and 4, and represented by Fig. 1.3, and 1.4 respectively. It can be seen that a small amount only of hydroxy acids exist in milk ceramides, which are present to quite a large extent in brain and serum ceramides, and which play an important role in relation to atherosclerosis⁽⁴³⁾. The types of ceramides that are considerably increased in atherosclerotic tissue, dead tissue, and in the event of stress^(43,44), are those which are rich in hydroxy fatty acids. The most biologically significant fatty acids have been found to be 2-hydroxylignoceric and 2-hydroxybehenic acids, which are absent from milk ceramide. Milk ceramides also have very little fatty acid of chain length greater than 18 carbons. Linoleic, oleic, palmitic and myristic acids are the major fatty acids present; the highly unsaturated nature of the ceramides (also attributable to unsaturated sphingosine bases, evidenced by the trans double bond in their infra-red spectra at 970 cm^{-1} - Fig. 3.4) would retard their mobility on TLC plates.

FIG. 1:3

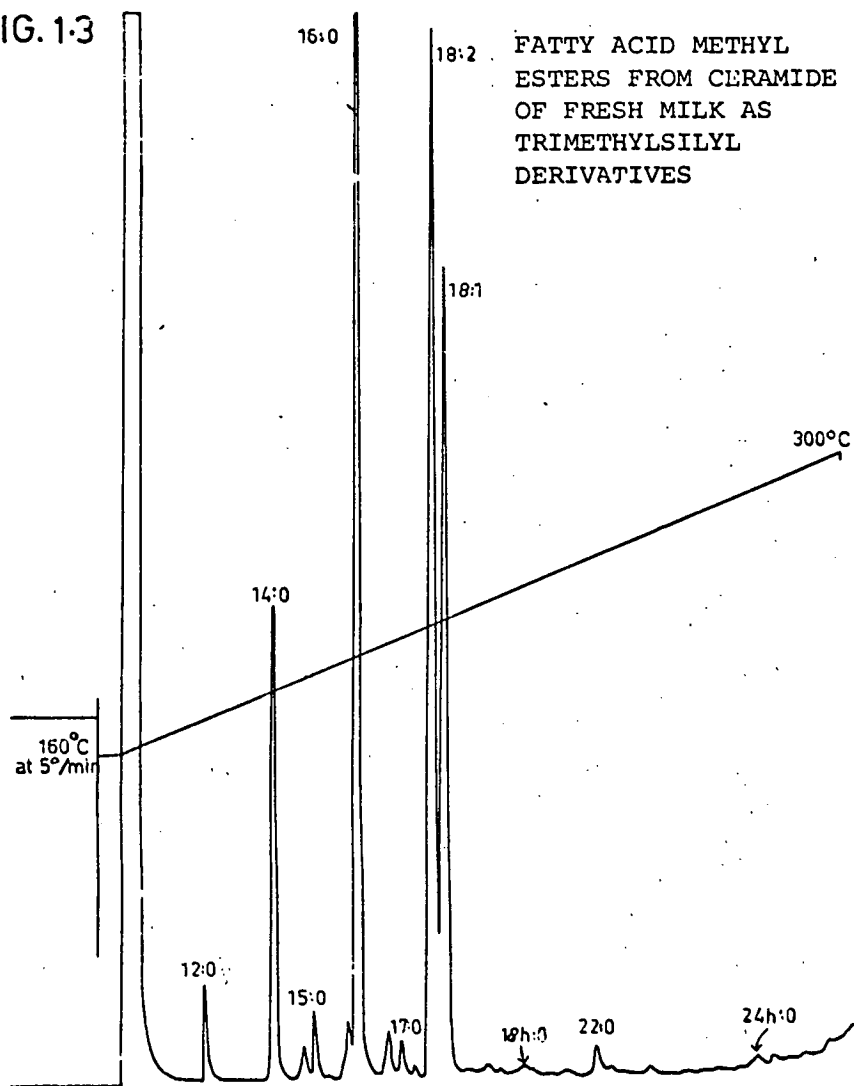
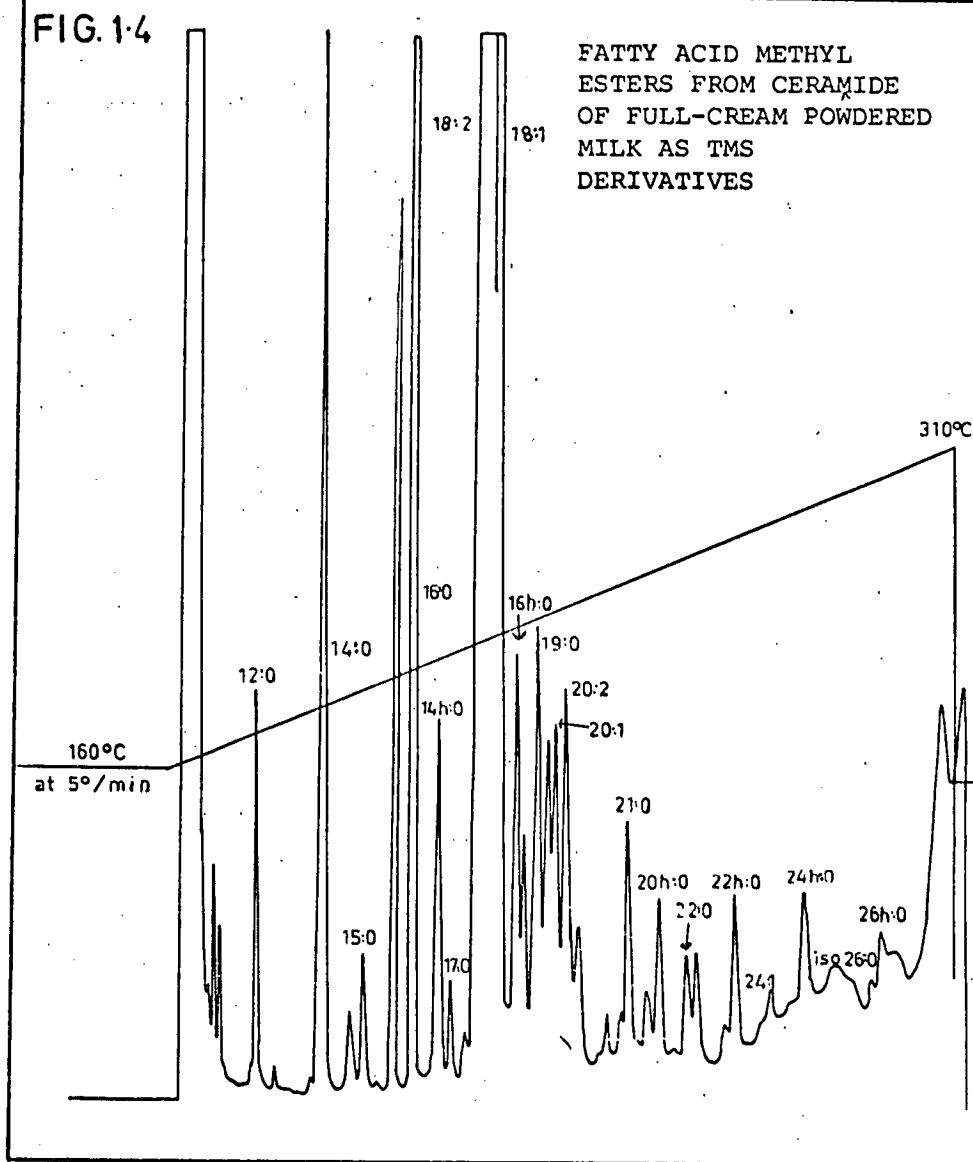


FIG. 1:4



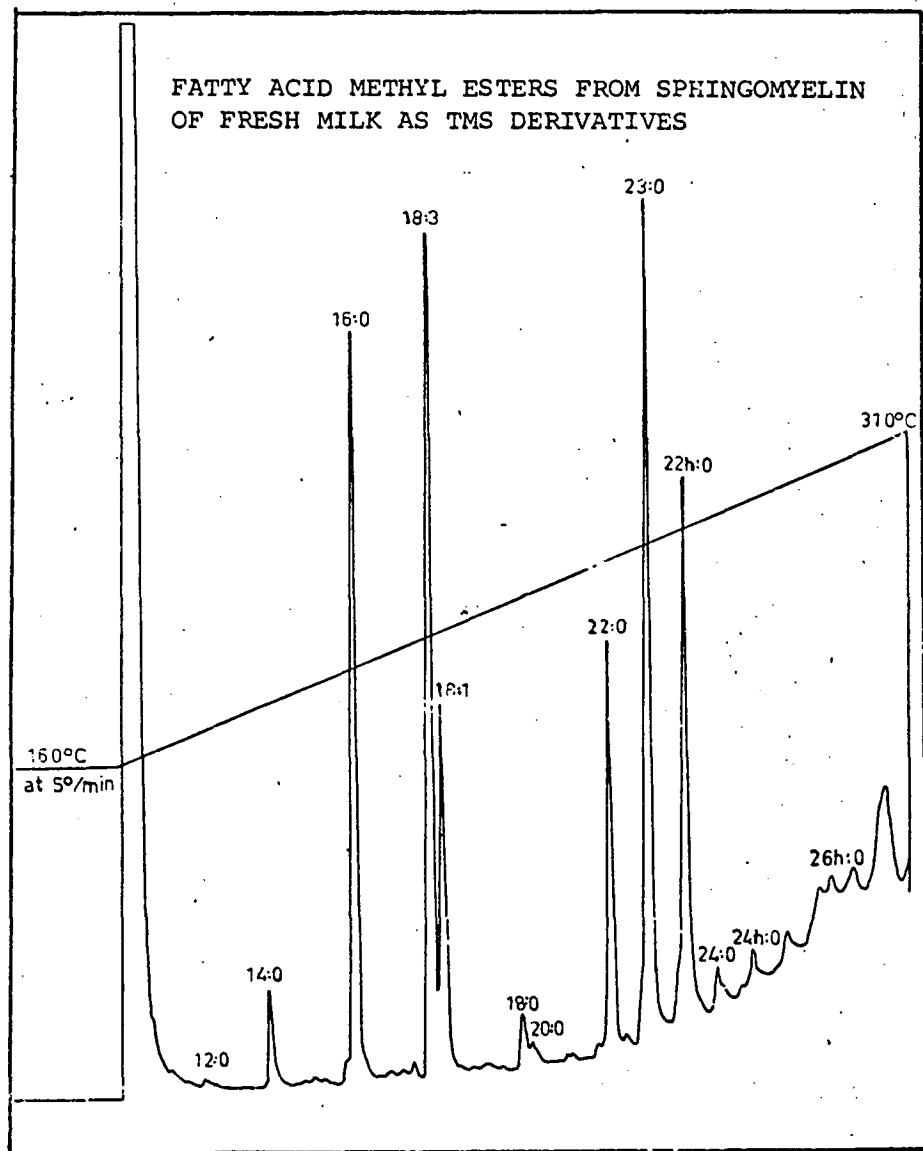


FIG. 1-5

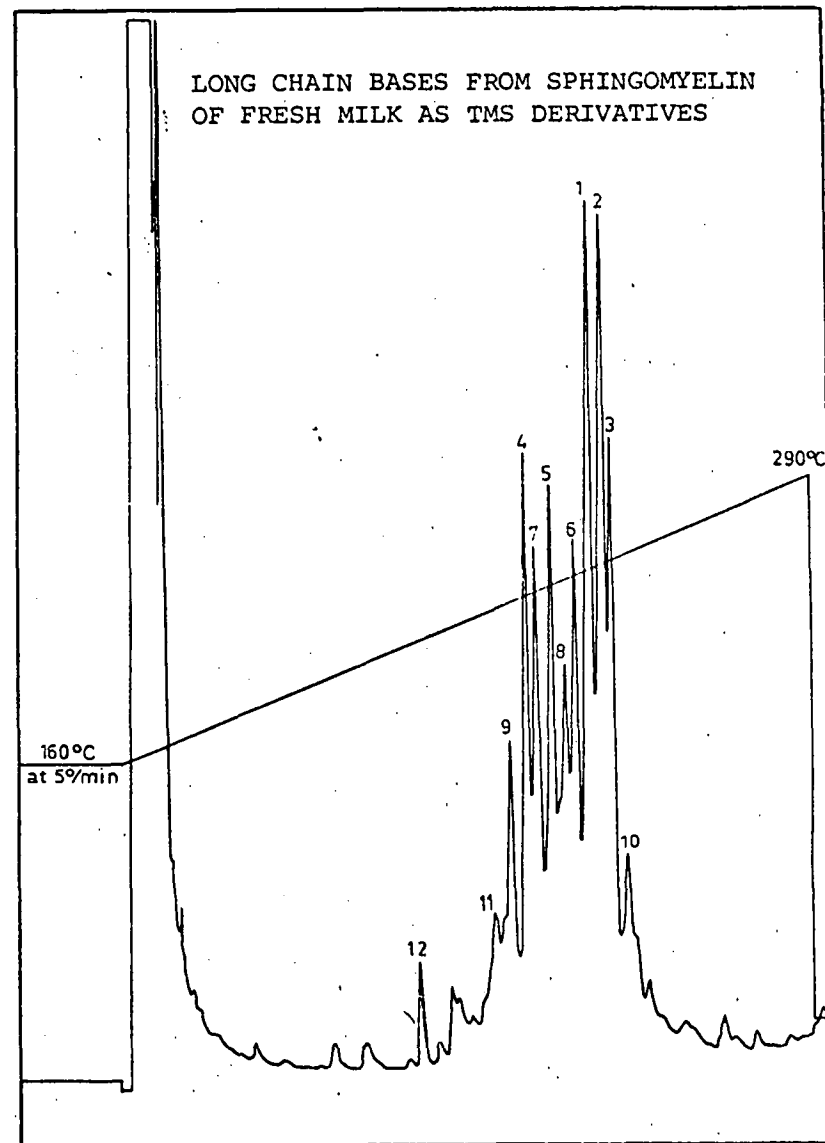
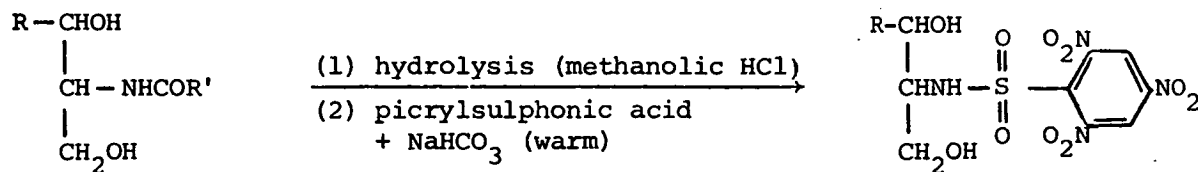


FIG. 1-6

Chapter Two

QUANTITATIVE METHODS FOR CERAMIDE ANALYSIS

Spectrophotometric Determination of Molar Quantities of Ceramide by Hydrolysis and Reaction with Picrylsulphonic acid



ceramide

The quantitation of glycosphingolipids has been carried out quite extensively by the reaction or complexing of some compound with the long chain base moiety; some methods make use of the fluorescence of a complex formed between the amine and a reagent such as 1-naphthylamino-4-sulphonic acid⁽²⁴⁾ or fluorescamine⁽²⁵⁾, while others are spectrophotometric such as the colorimetric estimation of the complex formed between the basic $-\text{NH}_2$ group of sphingosine and the anionic dye methyl orange⁽²⁶⁾. The following method describes the spectrophotometric estimation of the coloured reaction product formed between the long-chain bases of ceramide and trinitrobenzene sulphonic acid or TNBS (obtained from Sigma Chemical Co.). It has been suggested for ceramides and applied to glycosphingolipids in particular⁽²⁷⁾.

Hydrolysis of ceramide to long chain base fatty acid methyl esters was carried out by the method of Sweeley and Moscatelli⁽²⁸⁾, that is by refluxing the lipid (5-200 μg) in 1.5 ml of 2N methanolic hydrochloric acid (1 ml conc. HCl: 5 ml reagent grade methanol) in 10 ml glass tubes for 5 hours at 100°C (boiling water bath). By this method over 95% hydrolysis of ceramides could be achieved⁽²⁹⁾; it was found to be the most practical of several methods tried. Heating at 120°C for 90 minutes in 1.5 ml of 2N methanolic hydrochloric acid in a screw-capped Teflon-lined tube gave good results also,⁽²⁷⁾ but heating at hot-water

temperature (60°C) overnight gave much lower results at times, indicating incomplete hydrolysis. After cooling, 1.5 ml of water was added, and the long-chain bases extracted twice with 2 ml portions of chloroform, and the chloroform evaporated to dryness under a stream of nitrogen. 1 ml of sodium bicarbonate solution (4% aqueous) was then added to each tube, shaken vigorously to dislodge and suspend as much of the lipid as possible, and 1 ml of TNBS (1% aq.) added. The tubes were capped, and incubated one hour in the dark at 40°C, with occasional shaking. Methanolic HCl was then added (1N, 1 ml), and the solutions extracted twice with 2 ml portions of n-hexane, with vigorous shaking (200 times) to ensure good extraction.

The optical density of the yellow coloured sulphonates in 4 ml hexane was measured at 340 mμ against a reagent blank.

Table 2.1 gives the absorbances obtained for Koch-Light ceramides of concentration varying from 5 - 200 μg. Similar results were obtained for Sigma ceramides.

TABLE 2.1

Concentration, μg ceramide	optical density	
5	0.03	0.03
10	0.06	0.07
20	0.10	0.08
30	0.13	0.15
40	0.19	0.20
50	0.24	0.26
60	0.29	0.29
70	0.34	0.34
80	0.38	0.40
90	0.44	0.48
100	0.50	0.48
150	0.73	0.70
200	0.96	0.94

Figure 2.1 shows that Beer's Law is followed i.e. that the optical density is directly proportional to the concentration of the coloured compound in solution. The method was found to be accurate to a concentration of ceramide greater than 5 μg .

Extraction and Quantitation of Bovine Brain Ceramide from TLC Plates

Ceramide is a lipid of some polarity, by virtue of the presence of the two hydroxy groups and amido group of the sphingosine bases. Its chromatographic behaviour on wall-activated TLC plates shows this by the strong retarding of its motion; chemically bound water has been removed and possibly the O-H groups of the ceramide replace the sites where the H-O-H molecules were in the silica-gel matrix. The same effect is observed with cholesterol, and cerebrosides which have hydroxy groups also.

The following absorbance values show that 92% ceramide is extractable from TLC plates by cold or warm chloroform-methanol (2:1), but only about 20% is extractable by the non-polar solvent, chloroform. The ceramide (100 μg) is applied to the plate as a chloroform solution, the area containing the ceramide scraped off (approx. $\frac{1}{2}$ g silica gel), extracted three times with 5-10 ml solvent, the combined filtered extracts evaporated to dryness under nitrogen and the extracted ceramide determined by picryl sulphonic acid.

<u>Extracting solvent</u>	<u>Absorbance</u>
cold CHCl_3	0.04, 0.05
warm CHCl_3	0.05, 0.04
cold $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1)	0.34, 0.42
warm $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1)	0.37, 0.38
not extracted	0.40, 0.38
standard	0.44, 0.47

The values obtained for "not extracted" above apply if the amount of silica gel is small, so that the ceramide need not be extracted, but hydrolysis carried out in the presence of the silica gel. The latter remains mostly suspended in the aqueous phase when extracting the long-chain bases with chloroform.

A concentration series of ceramide (10-100 μg) was applied to TLC plates, the plates chromatographed in solvent A, and the four ceramide bands for each concentration scraped off the plate with a spatula after spraying with 0.1% aqueous eosin. The ceramide was hydrolysed⁽²⁸⁾ in the presence of the silica gel, and subsequently determined by picryl-sulphonic acid. Results are shown in Table 2.2

TABLE 2.2

Concentration (μg)	Absorbance				
	Band 1	Band 2	Band 3	Band 4	Total
10	.02	.05	.01	.02	.10
20	.04	.06	.03	.03	.16
40	.07	.12	.04	.06	.29
60	.10	.18	.05	.09	.43
80	.17	.22	.07	.11	.57
100	.19	.29	.09	.13	.70

The relative concentrations of the ceramides may be calculated by comparing the height of each curve (Fig. 2.2) for a particular concentration. In terms of percentage the four ceramide mixtures 1,2,3,4 are respectively in the ratio 18.6: 12.5: 41.8: 27.2, or approximately 1.5: 1: 3.3: 2.

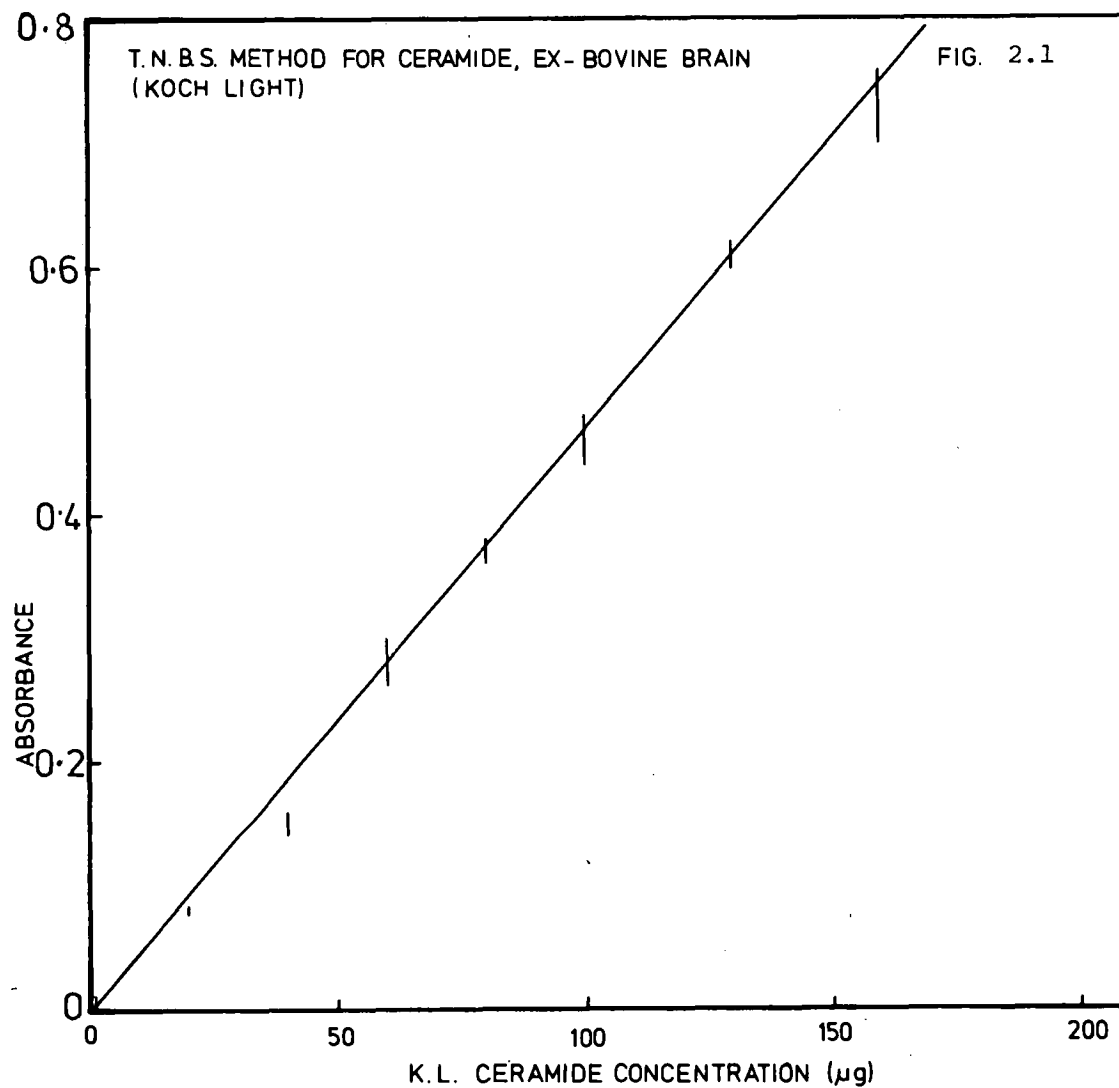
In Table 2.2 it is noted that the total absorbance obtained by adding the absorbances of the four ceramide bands for each concentration is higher than for the standard ceramide values in Table 2.1.

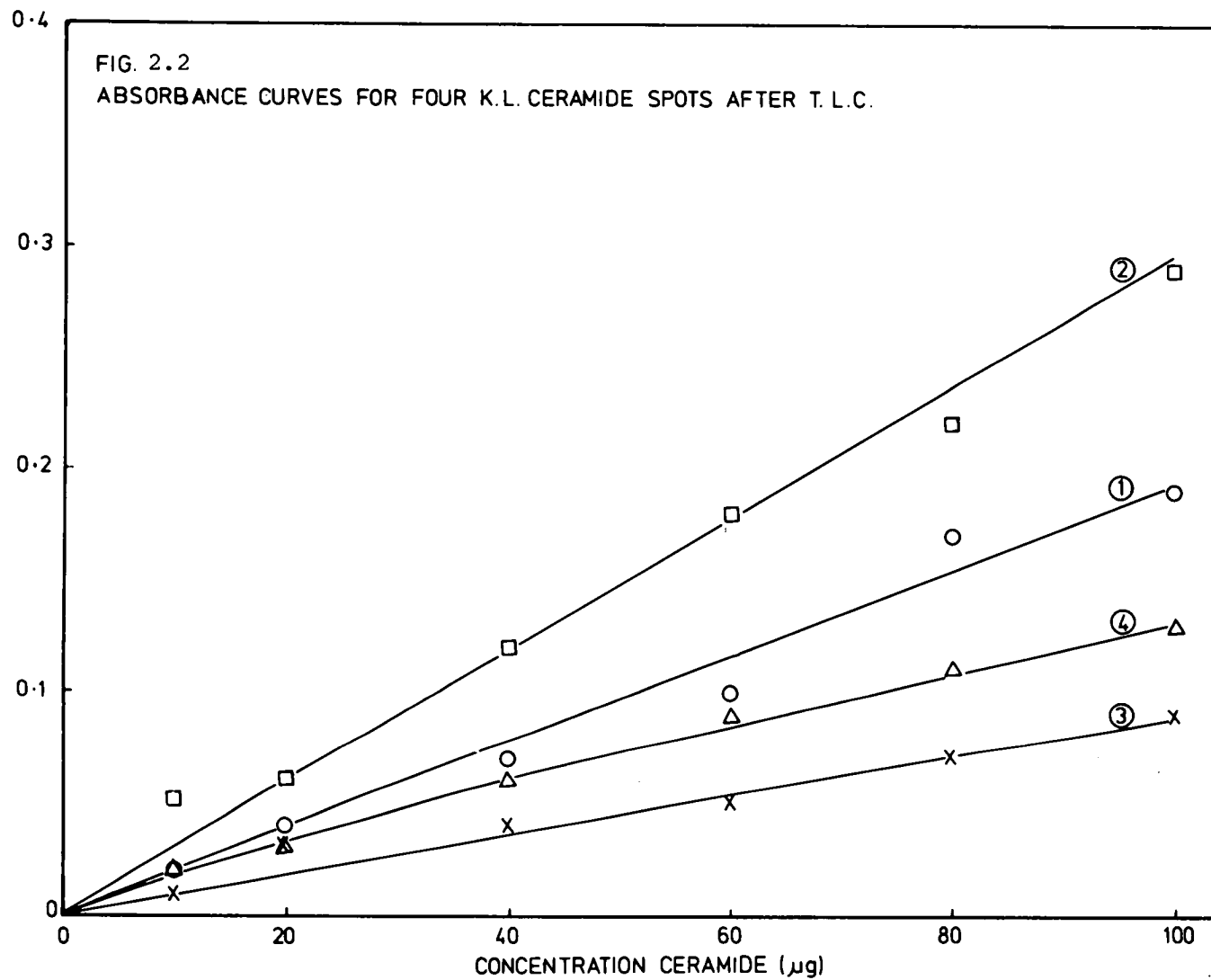
Estimation of total ceramide extracted from eosin sprayed plates

also showed higher absorbance, depending on the area of silica gel extracted. This was found to be due to the eosin indicator, which, though insoluble in chloroform and hexane, carries through in the picrylsulphonic acid method. A visible ultra violet scan shows that the indicator has some absorbance at 340 mμ.

The ratio 1.5: 1: 3.3: 2 was found by experience to vary somewhat with time. The lower two ceramides were initially of much higher concentration when a fresh solution of ex-bovine brain ceramides was prepared. However, at times, bands 3 and 4 became practically non-existent, and bands 1 and 2 increased much in concentration (plate 18.). The following conditions were found by TLC to have no immediate effect on the freshly prepared ceramide solution: concentration on TLC plates until overloaded; subjecting both oxygenated and nitrogenated ceramide solutions (chloroform) to ultra-violet light overnight; boiling for 10 minutes a solution of ceramide in chloroform.

The ceramide does decompose, however, when the solution becomes contaminated with the smoke produced when some of the lipid on the walls of the tube is charred. (columns 12-16). These solutions were tested for peroxides by shaking with freshly prepared KI-starch solution which turned blue within 3 minutes. Fresh ceramide solution contained no such oxidising power, and the blue colour was faintly observed after 30 minutes due to atmospheric oxygen.





Quantitative Ceramide Analyses of Milk Products

Several milk products were analysed for free ceramide by picrylsulphonic acid after separation of the ceramide from total lipid extract after TLC on preparative - scale plates. The exact method for obtaining the ceramide was as follows. A suitable quantity of the milk product (e.g. 5 ml fresh milk, 0.2g butter, etc.) was weighed or measured such that the butterfat content did not exceed 200 mg by too much and cause excessive overloading of the TLC plates; the amount of ceramide was very small, however, and the plates could have been overloaded to 300 mg of neutral fats. The total lipids were extracted by chloroform-methanol 2:1 by the method of Gloster and Fletcher,⁽³⁾ and, after filtering the extract through phase filter paper the solvent was recovered by evaporation under reduced pressure on a Rotavapor; the lipid residue was dissolved in 10 ml chloroform. The extracts were applied to preparative TLC plates (40 cm x 20 cm) by means of an automatic applicator, and the ceramides separated by chromatographing in solvent A. When the solvent front had reached nearly the top of each plate, it was removed from the tank, allowed to dry in a fume-hood 15 minutes, and the ceramide band (R_f - 0.4) located by spraying the edges and a strip in the middle of the plate with 0.1% aqueous eosin. Viewing under the U.V. light showed the ceramide fluorescing as a yellow band in the sprayed sections. This band was scraped off from the unsprayed portion of the plate, and the ceramide extracted with warm chloroform-methanol 2:1 and quantitated colorimetrically as the picrylsulphonate.⁽²⁷⁾ Results are tabulated in Table 2.3 showing ceramide in microgram (μ g) quantities per millilitre or gram of milk product. The quantity is also expressed as a weight percent on the butterfat in each milk product for a comparison of the relative degree of association of ceramide with butterfat.

TABLE 2.3

Milk Product (Butterfat ⁽¹⁸⁾)	Ceramide Concentration	% Ceramide ^{w/w} on Butterfat
Pasteurised milk (3/6%)	26µg/ml	0.07
Evaporated milk (8.3%)	60µg/ml	0.07
Full-cream powdered milk (27%)	140µg/g	0.05
Skim powdered milk (1%)	26µg/g	0.26
*Pasteurised skim milk (0.5%)	15µg/ml	0.30
Pasteurised cream (32%)	80µg/ml	0.025
Butter (83%)	150µg/g	0.02
Margarine (100% vegetable oil)	100µg/g	0.01

The ceramide concentration in whole-pasteurised milk is an average based on about 10 determinations carried out through the year. The result, 26 µg/ml is a little higher than that obtained for normal human blood serum, 20 µg/ml⁽²¹⁾. The relative percentage ceramide based on butterfat is observed to be similar for whole milk products i.e. whole milk, evaporated milk, and full-cream powdered milk; the latter gives a slightly lower result as does skim powdered milk compared with fresh skim milk, indicating some loss of ceramide in the drying process. Also, as the butterfat content increases in milk products, the relative percentage ceramide decreases very noticeably, as can be seen by comparison of skim-milk with butter or cream. This suggests that the ceramide is bound to the protein by hydrogen bonding and hydrophobic association in a similar manner to blood serum lipoprotein complexes.

*The skim milk was obtained by centrifuging whole milk, and drawing off the bottom layer. The fat content was determined by the Roesse-Gottlieb method (Jacobs M. p. 269⁽¹⁸⁾).

Attempts were initially made to determine ceramides in milk products without separation from other lipids by chromatography. These were found to give higher results, however, due to the addition to the yellow sphingosine picrylsulphonate of the yellow coloured fat-soluble carotenoid pigments. Also, other sphingolipids would react with the picrylsulphonic acid, especially sphingomyelin, which is 60% hydrolysed by the method used. The results obtained are as follows: pasteurised cows' milk (25µg/ml); human milk, 3 days after birth (50µg/ml); evaporated milk (80µg/ml); full-cream powdered milk (140 µg/g); butter (200 µg/g).

Quantitative Cholesterol Analyses of Milk Products

The method employed for total cholesterol determination is described on p. 360 by Kates,⁽³⁰⁾ and described in detail by Courchaine et al. (1959)⁽³⁰⁾ Free cholesterol may be determined by digitonin.

Reagents: Stock ferric chloride solution : 2.5 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in concentrated (85%) orthophosphoric acid and diluted to 100 ml with the same acid - stored in a brown glass-stoppered bottle at room temperature.

Ferric chloride colour reagent : 4 ml of stock ferric chloride solution diluted carefully to 50 ml with concentrated sulphuric acid, cooled, and stored at room temperature - discarded after two or three days as cloudiness arises.

0.5 ml aliquots of a chloroform solution of total milk lipids extracted from 0.5 ml milk were applied to preparative TLC fluorescent plates (silica gel GF 254), and the lipids separated using solvent A; for cholesterol analyses only, the plates could be well activated to retard the mobility of the cholesterol but not the triglycerides. The cholesterol plus ester bands were removed with a spatula, the lipids extracted with chloroform and the solvent evaporated to dryness under nitrogen. 6 ml of glacial acetic acid was added (burette) in which the

cholesterol was dissolved, and then 4 ml of ferric chloride colour reagent (burette) followed by mixing by inversion several times. The solutions were allowed to cool, and their absorbances read after 10 min. at 550 mμ against a reagent blank. A calibration curve was also plotted up to 1 mg of pure cholesterol; the reproducibility of this curve was checked from time to time using different samples of solvents of the same reagent grade. The calibration curve obtained was linear, and Beer's law was followed up to 1 mg. cholesterol (Fig. 2.3)

TABLE 2.4

Cholesterol Concentration (mg)	Absorbance
0.05	0.21, 0.21
0.1	0.46, 0.46
0.2	0.96, 1.00
0.3	1.46, 1.48
0.4	1.93, 1.94
0.5	2.42, 2.42
0.6	2.94, 2.97
0.7	3.44, 3.40
0.8	3.85, 3.87
Milk extract(0.5 ml)	0.52, 0.56, 0.50

Almost all the lipids present in milk lipids are triglycerides, and too much excess of these were found to interfere in the analyses, producing a cloudy solution, However, most or all of the triglycerides were separated free from cholesterol and its esters by TLC and the analysis carried out by the above method proved to be successful; a clear solution was obtained on reaction, with a characteristic purple colour of the reaction product.

The amount of cholesterol extractable from a TLC plate was also determined, at three different concentrations, 0.1 mg, 0.3 mg. 0.5 mg. The cholesterol solution was spotted on the plate and the area containing the lipid (about 5 cm²) removed with a spatula. The cholesterol was extracted carefully four times with small portions of cold chloroform.

Results:

Concentration (mg)	standard	Absorbance	
		extracted	% extractable
0.1	0.48, 0.47	0.44, 0.44	93%
0.3	1.42, 1.42	1.34, 1.35	95%
0.5	2.40, 2.38	2.25, 2.24	94%

Thus about 94% is extractable.

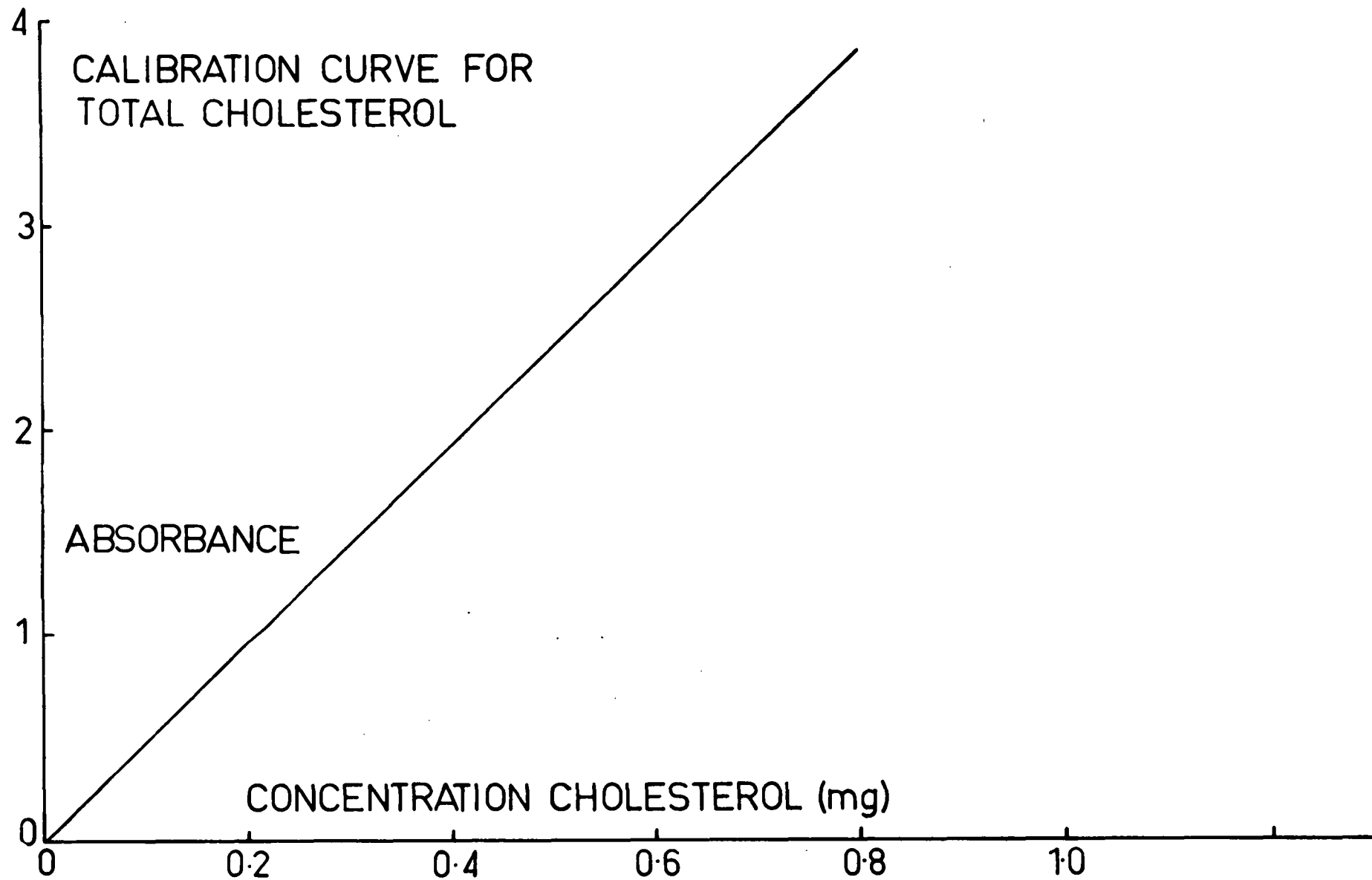
From these results, using the calibration curve, the concentration of cholesterol in fresh milk is 0.2 mg/ml.

The method appears to be reproducible; a calibration curve of say 0.1, 0.2, 0.3 mg cholesterol for each determination would ensure best results. The colour of the reaction product was observed to be reasonably stable for at least half an hour, after which time it began to deteriorate slightly. Most accurate results were obtained by reading the absorbance of each sample exactly 15 min after reaction i.e. in the same sequence as the reaction was performed.

Cholesterol in Ceramide

The presence of cholesterol was tested for in natural ex-bovine brain ceramides (Koch-Light) by spraying after TLC a plate containing 1 mg ceramide with ferric chloride spray reagent⁽³¹⁾ (50 mg FeCl₃·6H₂O in 90 ml water, 5 ml glacial acetic acid, 5 ml conc. H₂SO₄) and heating a few minutes at about 100°C. The TLC was carried out in two dimensions; (1) Chloroform, (2) solvent A. A faint purple spot at R_f (0.4, 0.8) was observed, and comparison with standard cholesterol spots indicated a concentration of 1 µg cholesterol/mg ceramide. A small amount of neutral lipid at the solvent front became apparent also as heating continued.

Fig. 2.3



Spectrodensitometric Analysis of Ceramide by Phosphomolybdic Acid

Few low concentrations of ceramides say less than 5 μg , a quantitative method of analysis based on transmission-densitometry of the lipid on the plate would probably be the most efficient and accurate when a large number of samples are to be analysed, given a good scanner or spectrodensitometer. Charring of total lipids has been examined by Rouser et al,^(5,6) steroids by Touchstone et al,⁽³²⁾ and serum ceramides by Z.A. Mejglo (Repatriation General Hospital, Hobart).⁽²¹⁾ The accuracy of this method depends on complete separation of components and uniform spraying of the plates with the sulphuric acid spray reagent. It has also been shown that the yield of carbon on charring is influenced by the degree of unsaturation of the component, its structure, and its mobility on the TLC plate. One advantage over the T.N.B.S. Method would be, however, that the quality of the spray reagent, sulphuric acid, is the same from sample to sample, whereas the quality of the picrylsulphonic acid reagent could vary slightly.

An interesting method of scanning charred lipids on TLC plates is to spray with "Neatan" plastic dispersion (Merck), remove the layer with transparent adhesive contact after drying, cut into strips and measure the transmittance through the charred spots. This method was met with some success, scanning the strips with a Spinco model RB Analytical recording densitometer and integrator ("Analytrol"), however, the filter in the instrument was suited only to scan blue-coloured spots, such as those obtained for serum proteins with bromophenol blue after electrophoresis. About 60% of the thin layer is removed on one application of "Neatan", a further 30-35% is removed on a second application to the remaining silica gel.

The use of phosphomolybdic acid has been applied to serum lipids⁽¹⁷⁾ and steroids,⁽³³⁾ the method depending on the blue reduced phosphomolybdate produced by the presence of unsaturation centres. The reagent was

used as follows to analyse chromatographically separated Koch-Light ceramide (ex-bovine brain). Nearly linear calibration curves were obtained for concentrations between 30 μg and 100 μg . The "Analytrol" was used for scanning, with a slit width of $\frac{1}{2}$ mm.

Either clear plastic or very thin glass sheets could have been adopted for the experiments; some 20 cm x 20 cm translucent plastic sheets were available that were inert to reagents and TLC solvents. These were convenient as they could be cut into strips before or after TLC for use in the Analytrol.

The plastic plates were coated with a thin layer of silica gel G, air-dried 30 minutes, activated at 110°C for 17 minutes, and cooled 30 minutes in the dessicating cabinet. The plates were removed, and the silica gel divided accurately into six strips 3 cm wide; the silica gel was scraped off lengthwise from the plates on each dividing line with a narrow spatula sharpened to suit the purpose (ca. 2 mm wide), so that a silica gel strip 2.8 cm wide remained. Ceramide solution (10 mg/ml) was now applied to the plates by a 10 μl syringe 5 cm from the bottom as a thin line 1.0 cm wide in the centre of each strip. The concentrations used ranged from 5 μg to 200 μg . The plates were developed chromatographically in solvent A, air-dried, and sprayed as evenly as possible with a 10% methanolic solution of phosphomolybdic acid. A very fine spray was used, and the plates were not allowed to become too wet with methanol as the ceramide could spread. The plates were then cut carefully into strips with sharp scissors so as not to disturb the edges of the silica gel impregnated with phosphomolybdic acid; even any slight disturbance would render the determination inaccurate. Usually the silica gel layer had become separated from the plate in the region of the solvent, and the first 4-5 cm broke up and fell off before or during cutting. Thus the silica gel to the line of application was removed deliberately before cutting. The strips were then placed on a

glass plate in an oven at 105°C-110°C for 4 minutes, and air cooled 5 minutes before scanning by the Analytrol. The region of the strip just above the first ceramide was used to zero the instrument. Results for concentration vs. area under the curve are shown in Table 2.5 and graphically illustrated by Fig. 2.4. Also results for total area under the curve vs. time of heating (at 100-105°C) are tabulated and graphed. The optimum temperature of 100-110°C had been approximately determined from preliminary experiments. Less than 100°C caused slow reaction and dulling of the surface.

TABLE 2.5

Ceramide		Area under curve by Analytrol (cm ²)								
Concentration (µg)	ceramides 3 & 4			ceramide 2		ceramide 1			Total	
5	0.4			0.3		0.2			0.9	
10	0.8	0.7		0.6	0.5	0.2	0.2		1.6	1.4
15	1.2	0.8	0.7	0.4	0.3	0.23	0.4	0.3	1.9	1.5
20	1.4	1.25	1.5	0.3	0.3	0.8	0.3	0.45	0.4	2.0 2.0 2.7
25	1.5			0.5		0.8			2.8	
30	1.6	2.0		0.6	0.5	0.8	0.3		3.4	2.4
40	2.2	1.6		0.6	0.8	1.0	0.6		3.8	3.0
45	1.3			0.4		0.3			2.0	
50	2.7	2.0		1.0	0.7	1.1	0.9		4.8	3.6
60	2.8	1.7	1.7	1.0	0.8	0.7	1.0	1.2	0.6	4.8 3.7 3.6
70	2.5			0.9		0.8			4.2	
80	3.5	3.0		0.9	1.2	1.3	0.9		5.7	5.1
90	2.8			1.0		1.5			5.3	
100	3.0	1.6		1.2	0.5	1.6	0.9		5.8	3.0
150	4.5	2.3		1.5	1.3	2.0	1.8		8.0	5.4
200	7.0	4.0		3.5	2.1	2.9	3.6		13.4	9.7

TABLE 2.6

Time of heating of 50 μ g ceramide @ 100-105°C (min)	Total area under curve by Analytrol	
1.0	1.4	1.6
1.5	3.0	2.5
2.0	3.7	4.2
3	4.5	4.7
4	4.6	4.9
5	5.05	4.7
7.5	4.75	4.85
10	4.3	4.55
15	3.1	3.6
30	2.2	2.6

A study of the graphs shows that above 100 μ g, the results are not very reliable at all, but over a limited range a reasonable linear calibration curve may be obtained. There are many variables, however, as outlined in the following paragraph.

Heating time and temperature are to be kept constant, as determined by experiment (105°C for 4.0 minutes was used). The 1 cm line of application of the ceramide is to be kept as narrow a width as possible (1.5 mm). If the ceramide bands are inclined at too great an angle they cause overlapping of peaks on the analytrol. It was found that this was the case if the silica gel was of an uneven thickness. The strips could only be used once since to use them again would mean that they would have to be overlapped on the spreader and cause uneven thickness. The separation of the four ceramide bands affects the results also, the peak heights decreasing and overlapping as R_f values decreased. Even at the best condition of activation of the plates complete separation of ceramides 3 and 4 could not be affected in the solvent used for relatively large concentrations (>20 μ g).

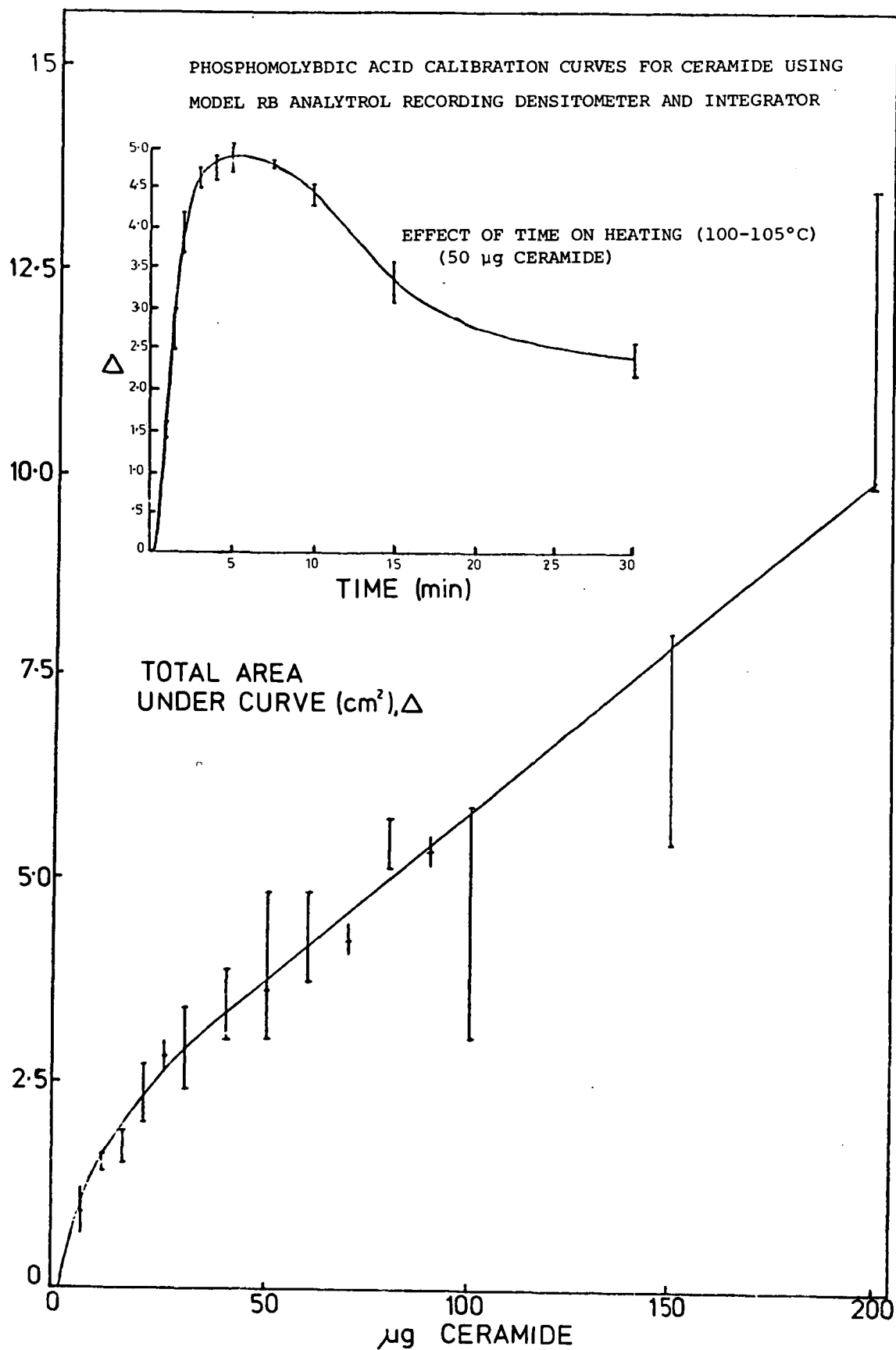


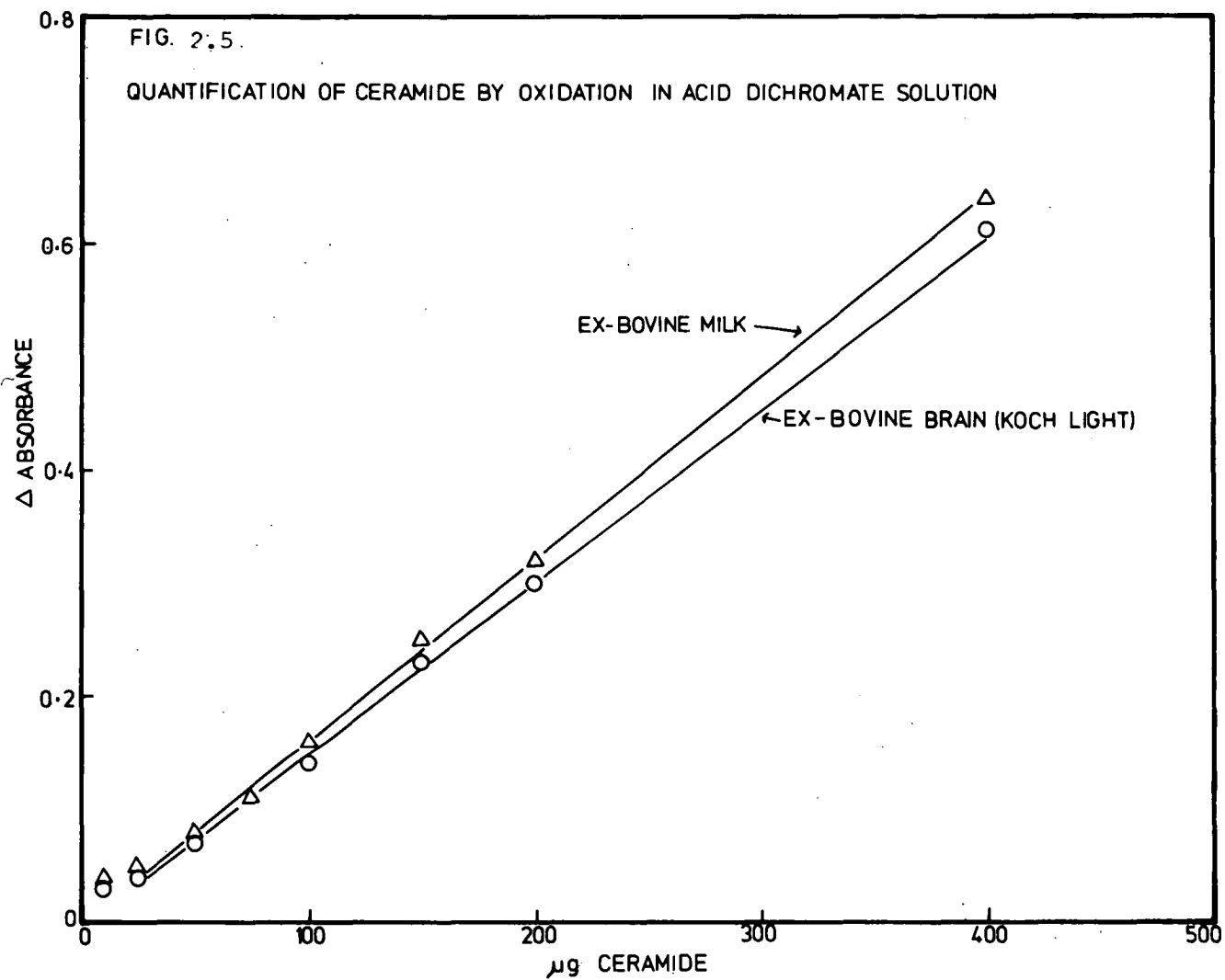
FIG. 2.4

Colorimetric Determination of Ceramide by Reduction of Acid-Dichromate

The ability of various lipid classes to reduce acid-dichromate solution quantitatively has been proposed as a method⁽³⁴⁾ and applied to the quantitation of cholesterol, cholesterol stearate, tripalmitin, palmitic acid and lecithin.⁽³⁵⁾ Its application to ceramides is here described, with some analyses carried out on milk.

Microgram quantities of ceramide (to 200 μg) were heated with 3 ml of acid-dichromate reagent (2.5g $\text{K}_2\text{Cr}_2\text{O}_7$ /1 of 36N H_2SO_4) for 45 min. in glass stoppered tubes in a boiling water bath. The reduced solutions were then cooled, diluted with water (40x) and the absorbance read at 350 m μ against a blank of equimolecular potassium chromium sulphate, or water since its absorbance is practically negligible at 350 m μ . The change in absorbance, i.e. the amount of dichromate reduced, was found to be linearly proportional to the amount of ceramide above 10-20 μg . (Fig. 2.5). Koch-Light ceramides and fresh milk ceramides were used directly for the standard curves shown. The milk ceramides had been obtained previously from column and preparative-thin-layer chromatography on the lipids extracted from fresh milk; these were slightly contaminated with some ceramide derived from the hydrolysis of milk sphingomyelin. It is noticed that the curve for the milk ceramides has a higher gradient than that for the brain ceramides. This could be just a concentration error, or due to the relative nature of the fatty acids; a high proportion of hydroxy fatty acids are present in brain ceramides, but the relative amount of unsaturated fatty acids in milk ceramide compared to brain ceramide is high, and mostly linoleic (details later). Unsaturated fatty acids will reduce more dichromate as the oxidation of one double bond requires 4 (O), whereas the oxidation of secondary alcohols requires only 1 (O).

Separation of ceramide from milk lipids extracted from 1 ml milk on a preparative TLC plate that has been previously washed once with the



chromatographing solvent (solvent A) yields a value of 20 $\mu\text{g/ml}$ by the above method of analysis.

Experience could bring the accuracy of this method to 5-10 μg ceramide, as the picrylsulphonic acid method has been. However, for quantities less than 5 μg , a more sensitive method must be found. Perhaps the addition of some organic compound, such as an aromatic aminophenol, may increase the colour and thus sensitivity of the above method.

Separation of Ceramides from Neutral Lipids by Partition Between Solvents

The following procedure has been used to separate to greater than 97% polar lipids nearly completely from neutral lipids.⁽³⁶⁾ The distribution is carried out in two separatory funnels by the single withdrawal procedure using the solvent system petroleum ether (40-60) -87% ethanol.

2 mg of Koch-Light ceramides were dissolved in 25 ml of the petroleum ether (upper) phase (equal volumes of petroleum ether and 87% ethanol pre-equilibrated), and the solution transferred to a separatory funnel (50 ml) and shaken with 10 ml of the lower ethanol phase. After five minutes the equilibrated lower phase was transferred to a second funnel containing 25 ml of upper phase, and a further 10 ml of lower phase added to the first funnel. Both funnels were shaken, the lower phase of the first funnel transferred to the second one. This partitioning procedure was repeated six times, and all the ethanol phases and petroleum ether phases analysed for ceramide content by picrylsulphonic acid.

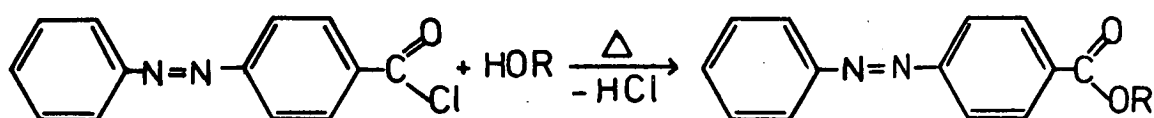
The results are given in Table 2.7.

TABLE 2.7

Ethanol Phase	Absorbance	Pet. ether Phase	Absorbance
1	1.22	1	2.48
2	1.36	2	<u>2.82</u>
3	1.28		<u>5.30</u>
4	1.21		
5	1.08		
6	<u>0.91</u>		
	<u>7.06</u>		

The total absorbance above (12.36) corresponds to absorbance for 2,000 μ g of K.L. ceramides, from Fig. 2.1. Only 57% of the ceramide is extracted on 6 partitioning steps, but the method could be useful still as more extraction could be carried out, say, to 12 extractions. The method is quick and previous reports indicate that only 0.02-0.03% neutral lipids contaminate the polar phase on six extractions.

Coloured Derivatives of Ceramides



Identification and chromatographic separation of many amines and alcohols has been carried out by reaction of the functional group with a highly coloured reagent. Woolfolk et al⁽³⁷⁾ used p-phenylazobenzene-sulphonyl chloride and Amin⁽³⁸⁾ used p-nitrophenylazobenzoyl chloride to identify amines. Alcohols (normal, secondary and isoalkyl) have been identified as the red to orange coloured p-phenylazobenzoates up to ten carbon atoms by Woolfolk et al⁽³⁹⁾. In the following experiments

the same reagent was used (p-phenylazobenzoyl chloride) to esterify various alcohols met with in lipid work, including cholesterol and Koch-Light ceramides.

A mixture of the acid chloride (0.1g), alcohol (excess) and 3 ml of pyridine were refluxed gently for 1 hour. The red solution was poured with vigorous stirring into ice and 10% sodium bicarbonate solution and left to stand a few minutes for complete crystallization of the ester. The ester was filtered, washed with water and dried in a dessicator overnight. It was then dissolved in a small volume of petroleum ether (60-80)-benzene and chromatographed on a column (10 cm x $\frac{1}{2}$ cm) of activated neutral alumina on which the free acid was strongly adsorbed. The ester was eluted with up to 10% benzene in petroleum ether, and finally recrystallized from absolute ethanol or ethanol-water. Melting points and yields of the esters are tabulated as shown in Table 2.8; the esters were not microanalysed for their elements.

TABLE 2.8

Melting points and yields of p-phenylazobenzoates

Alcohol used	Melting point (°C)	Yield %
1-octanol*	57-58	54
1-hexadecanol	67-68	53
glycerol	133-135	43
ethylene glycol	124-126	63
cholesterol	188	50
ceramide	70-100	40

*Prepared by Woolfolk et al., m.p. 56.3-57.6, yield 68%

All derivatives prepared were soft, fine needles, except for ceramide, which was a brittle solid. The infra-red spectrum of the ceramide

derivative showed the amide C=O and N-H bands at 1630 and 1530 cm^{-1} , as well as the ester carbonyl absorption in the 1720 - 1730 cm^{-1} region.

Chromatography of the derivatives on silica gel using non-polar solvents hexane, diethyl ether, dioxane showed R_f values increasing in the order of octyl, cetyl and cholesteryl esters with ceramide and acid chloride at the solvent front. The solvents used were previously dried (Na), and Na_2CO_3 used to remove free acid from the chloroform solutions of the derivatives. On a plate containing 0.5% Na_2CO_3 , and using hexane-diethyl ether 9:2, the following R_f values were obtained: octyl ester 0.90; cetyl ester 0.93; cholesteryl ester 0.95.

The formation of coloured derivatives of ceramides (and cholesterol) similar to above could be useful for colorimetric analysis of these lipids. The p-nitrophenylazobenzoyl chloride (obtainable from Eastman Chemical Co., as are the other reagents) is a very highly red coloured compound, and its reaction with sphingosine bases after hydrolysis of ceramide may give a colorimetric method of determining ceramide less than 5 μg .

Chapter Three

SYNTHETIC CERAMIDES AND SPECTRAL ANALYSIS

Synthesis of Ceramides

The synthesis of ceramides has been carried out by a variety of methods, mostly depending on acylation of the long-chain base by an acid chloride to give a di-O-acyl-N-acyl compound, followed by mild alkaline hydrolysis to cleave the more labile O-acyl linkages⁽⁴⁵⁻⁴⁷⁾. Selective N-acylation has been achieved, however, with acyl chlorides in N,N-dimethylformamide-pyridine⁽⁴⁸⁾ or acetic anhydride in methanol⁽⁴⁹⁾. The following methods of Ong and Brady⁽⁵⁰⁾, and Hammarstrom⁽⁵¹⁾ are more direct and were used to study the products of ceramide synthesis by TLC, infra-red spectroscopy and mass spectroscopy.

The synthesis of ceramides using N-hydroxysuccinimide esters was carried out by direct N-acylation of DL-sphinganine (mainly DL-erythro-1, 3-dihydroxy-2-aminooctadecane, obtained from Koch-Light Laboratories Ltd.) The esters were synthesised in two steps: N-hydroxysuccinimide was prepared by reacting succinic anhydride with N-hydroxylamine hydrochloride by the method of Anderson et al.⁽⁵²⁾ The product obtained (45 g white crystals, 40%) after one recrystallization from ethyl acetate melted at 197°C. This was used to prepare the esters, by condensation with fatty acids in dry ethyl acetate in the presence of a carbodiimide⁽⁵³⁾ (dicyclohexylcarbodiimide was used). The esters were recrystallized from ethanol, and shown to be chromatographically homogenous by TLC on silica gel G using chloroform as the developing solvent. The dicyclohexylurea byproduct was recrystallized (mp 215°C) from ethanol and stored in the cold for recycling. The melting points and approximate yields for the prepared esters were as follows: palmitoyl ester, 88°C, 90%; oleoyl ester, 117°C, 70%; linoleoyl ester, 117°C 60%; ricinoleoyl ester 119°C, 80%.

N-palmitoyl sphinganine and N-linoleoyl sphinganine were prepared by reacting 2 mg of DL sphinganine with 2 mg of the N-acylsuccinimide in 1 ml of tetrahydrofuran; the solution was left overnight at room temperature in capped tubes, and heating slightly for several hours

after this time did not increase the yield significantly as shown TLC. The solution was evaporated to dryness, the residue dissolved in 1 ml chloroform and used for spotting. The results are shown on photograph 19 (page 65). By TLC analysis alone, 4 spots are observed for the N-linoleoyl sphinganine at R_f values of 0.10, 0.35, 0.40, 0.43, and 5 spots for N-palmitoyl sphinganine at R_f values of 0.10, 0.16, 0.24, 0.31, and 0.43. These results indicate that different structural forms of the same ceramide could exist, or the ceramide in association with some reaction-by-product on the plate.

The method of Hammarstrom⁽⁵¹⁾ was used to prepare ceramides in sufficient quantity to be able to isolate the different bands obtained on TLC and subject them to analysis by spectral methods for partial characterization at least. The following ceramides were prepared; N-oleoyl DL-sphinganine; N-linoleoyl DL-sphinganine; N-hexanoyl DL-sphinganine (the crude ceramide of this short fatty acid chain type was prepared by Dr. A.J. Blackman); N-palmitoyl D-sphingenine. The latter ceramide was prepared in minute quantities only, but a TLC analysis reveals the same number of ceramide bands (6 for a fine analysis), and in positions with similar R_f values (lower in each case, since a sphingenine derivative), as the sphinganine derivatives. A TLC analysis is tabulated later. The following method describes the preparation of a ceramide by direct coupling of the long-chain base with a fatty acid in the presence of a mixed carbodiimide; the latter causes activation of the carboxylic acid to a mixed carboxylic-ammonocarboxylic anhydride which then reacts with the amine. 30 mg of DL-sphinganine (100 μ mole) and 60 mg of fatty acid (200 μ mole) were dissolved in 15 ml of CH_2Cl_2 , 15 ml of CH_3CN and 3 ml of CH_3OH ; the dichloromethane was initially washed with water to remove phosgene, dried over calcium chloride and distilled for

immediate use. 100 mg of N-ethyl-N-(dimethylaminopropyl) carbodiimide in 7 ml of CH_2Cl_2 was added, the flask stoppered and incubated overnight at 40°C . The solution was transferred to a 1 litre separatory funnel with 300 ml of diethyl ether, and the mixture washed with 3 x 90 ml of 5% NaHCO_3 , once with 15% NaCl , with 3 x 90 ml of 1N HCl , and then with 3 x 90 ml of 15% NaCl to bring to neutral (the acid wash could be omitted maybe, so as to recover unreacted long-chain base). The ether extract was washed twice more with distilled water, dried over anhydrous magnesium sulphate, a few mls of absolute ethanol added, and the ether evaporated in vacuo. The residue was taken up immediately in chloroform and analysed for ceramides by TLC in solvent A. (Figs. 3.1, 3.2). Table 3.1 gives the R_f values of the ceramide bands for the prepared derivatives, compared to Koch-Light ceramides.

TABLE 3.1

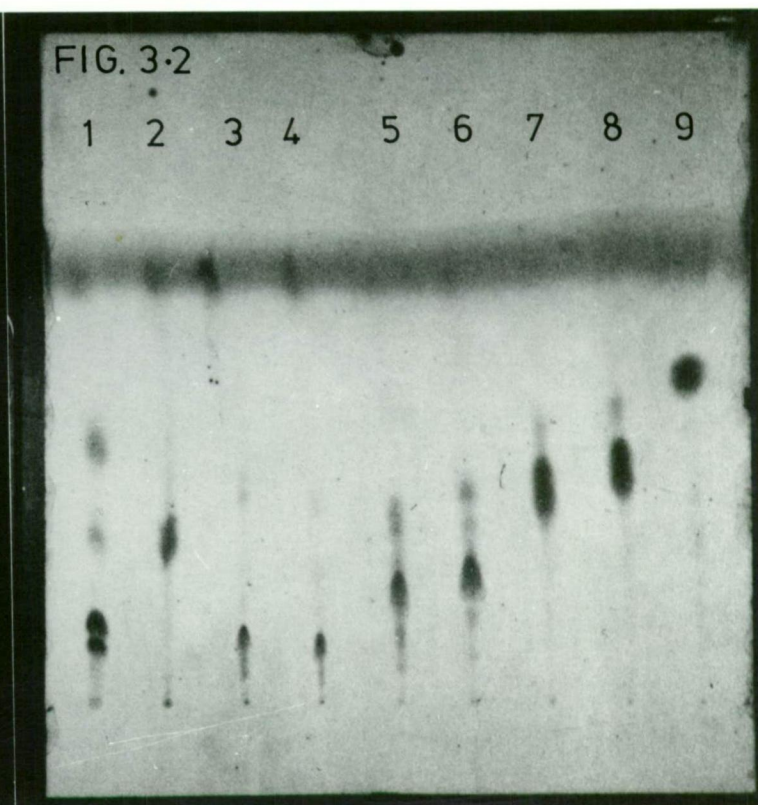
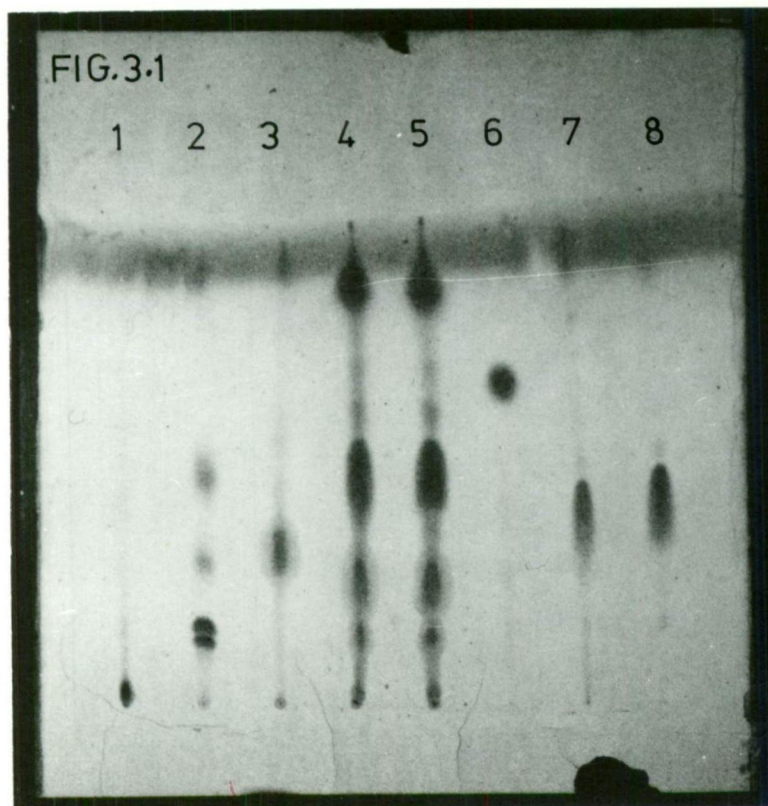
Koch-Light	N-palmitoyl-D -sphinganine	N-oleoyl DL sphinganine N-linoleoyl DL-sphinganine	N-hexanoyl* DL-sphinganine
	0.62	A. 0.69	
	0.52	B. 0.60	
Band 1 0.50	0.47	C. 0.51)	
Band 2 0.30	0.40	D. 0.43)	0.33 (pure)
Band 3 0.17	0.25	E. 0.27 band 2	
Band 4 0.14	0.10	F. 0.15 band 3	

*The crude preparation of N-hexanoyl DL-sphinganine is represented on photograph 20 (p. 65).

The major bands of N-oleoyl and N-linoleoyl sphinganine on the TLC plates are designated bands 1, 2, and 3, respectively down the plate. The crude mixtures of both ceramide preparations were applied to preparative TLC 40 cm plates, and the individual bands recovered

quantitatively. The recovered quantities and yields were, for both ceramides: band 1, 30 mg (50%); band 2, 4 mg (7%); band 3, 6 mg (10%); thus a total of 67% ceramide was recovered, in agreement with the expected 60-75% (51).

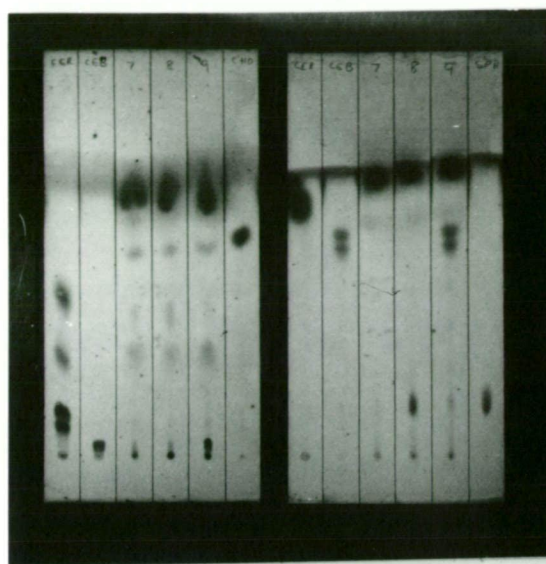
The 6 bands, 3 for each ceramide preparation shown on Fig. 3.1 are represented on Fig. 3.2 after separation on thick layer plates and recovery. Band 1 (a mixture of spots C and D - see Table 3.1) for both ceramides is observed to be practically pure, with a very slight contamination with spot B. Band 2 for each ceramide contains a little impurity, which appears to fatty acid mainly. Band 3 for each ceramide is virtually free from any contamination, as seen by the single spots on the chromatogram (R_f 0.15). However, an interesting observation is also made, that has been observed previously for naturally occurring Koch-Light ceramides (ex-bovine brain). Faint spots appear in the region corresponding to band 1, suggesting the possibility of some difference in the molecular structure of the ceramide, thus causing it to be retarded on the TLC plate. For Koch-Light ceramides the similar observation has been the shift of the ceramides from bands 3 and 4 to bands 1 and 2. Dr. Polya suggested that these observed anomalies could be due to the formation 1,2- or 1,5- epoxides, or heterocyclic ring structures containing O and N such as in oxazolines. All bands, both naturally occurring and synthetic, gave rise to intensely blue-coloured spots by the *t*-butyl hypochlorite KI-starch test, indicating a primary or secondary amino group available for chlorine substitution.



From left to right:

- (1) DL-sphinganine
- (2) Koch-Light ceramides
- (3) N-hexanoyl DL-sphinganine (N)
- (4) N-oleoyl DL-sphinganine (O)
- (5) N-linoleoyl DL-sphinganine (L)
- (6) Cholesterol
- (7) Oleic acid
- (8) Linoleic acid

- (1) Koch-light ceramides
- (2) N-hexanoyl DL-sphinganine
- (3) Band 3 (O)
- (4) Band 3 (L)
- (5) Band 2 (O)
- (6) Band 2 (L)
- (7) Band 1 (O)
- (8) Band 1 (L)
- (9) Cholesterol



17

(CER) Ceramide; (CEB) Cerebroside;
(CHO) Cholesterol; (SPH) Sphingo-
myelin; (7) Milk incubated 3 days;
(8) As for 7, with sphingomyelin
added; (9) As for 7, with cerebroside
added.

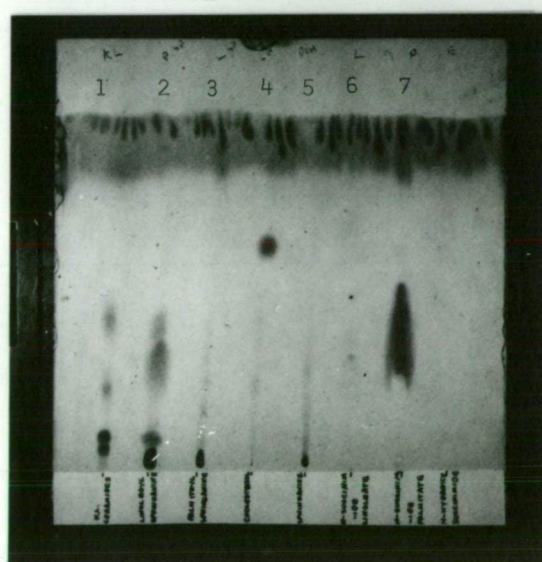
Left - sol. A.

Right - sol. B.



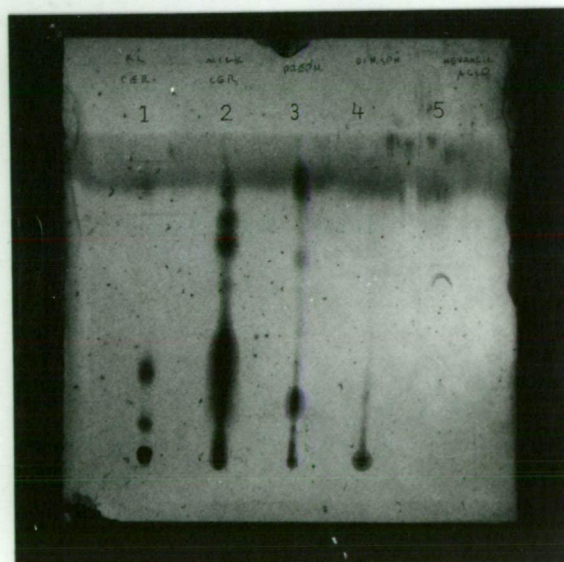
18

(1,2,3,6,8,11) Old ceramide samples;
(4,5,7) New samples; (9) Fresh
sample boiled; (10) Sunlight for 1
day on old sample; (12) U.V. light
on old sample 3 hours; (13-16)
Samples heated overnight (65°C).



19

- (1) Koch-Light ceramide
- (2) N-linoleoyl DL-sphinganine
- (3) N-palmitoyl DL-sphinganine
- (4) Cholesterol
- (5) DL-sphinganine
- (6) N-linoleoylsuccinimide
- (7) N-palmitoylsuccinimide



20

- (1) Koch-Light ceramide;
- (2) Milk ceramide (overloaded);
- (3) N-hexanoyl DL-sphinganine (syn.);
- (4) DL-sphinganine
- (5) Hexanoic acid

Infrared Spectra of Synthetic and Naturally Occurring Ceramides

The general structures of the synthetic ceramides, i.e. N-hexanoyl-sphinganine (Fig. 3.14) and the 3 bands each of N-oleoylsphinganine and N-linoleoylsphinganine (Figs 3.8-3.10) were determined by their infrared spectra in carbon tetrachloride ($\sim 0.5-2$ M), and compared to naturally occurring bovine brain and milk ceramides (Figs. 3.3-3.7). Absorption bands common to all spectra are as follows (data derived from Conley⁽⁵⁴⁾). O-H and N-H stretching vibrations occur generally as a broad absorption band between 3700 and 3100 cm^{-1} . The amido group is indicated by the presence of absorption bands at $1650-1630\text{ cm}^{-1}$ (amide band I: C=O stretch), $1550-1530\text{ cm}^{-1}$ (amide band II: in plane N-H deformation strongly coupled with C-N stretch; also O-H bend); a strong C-O stretch occurs at $1070-1040\text{ cm}^{-1}$, (amide band III). The asymmetrical C-H bend of medium intensity occurs at 1470 cm^{-1} (CH_2 , CH_3), and a weak symmetrical C-H bend at 1380 cm^{-1} (CH_3).

Absorption bands other than those given above that characterize ceramides are due to double bonds, both cis - (of the fatty acid moiety), and trans-(of the sphingosine long-chain base moiety). The C-H out-of-plane deformation for the trans double bond occurs as a strong band at 970 cm^{-1} (Figs. 3.3-3.7). This band is very intense relatively for ex-bovine brain ceramide band 2 (Fig. 3.6), and it is coupled with a strong C-H in-plane deformation band at 1300 cm^{-1} , which is much less intense for the other three bands. This indicates a relatively high sphingosine (mostly D-erythro-trans-1, 3-dihydroxy-2-amino-4-octadecene in animal sphingolipids) content in brain ceramide band 2, compared to the other ceramide bands, in which the double bond could be epoxidized in part thus reducing the C-H deformation due to the trans double bond. A weak band also occurs for the naturally occurring ceramides at 3000 cm^{-1} , due to the asymmetric C-H stretch of both cis and trans double bonds.

This band is observed to be of strong intensity in the N-linoleoyl-sphinganine due to the 2 cis double bonds per molecule (Figs. 3.8-3.10), of medium intensity in the N-oleoylsphinganine (not shown but the spectra are practically identical to the corresponding N-linoleoyl sphinganine) and non-existent in N-hexanoylsphinganine (Fig. 3.14). The C=C stretch is observed between 1650 and 1680 cm^{-1} for both cis and trans configurations, mostly masked by the amide I band.

The O-H, N-H coupled stretch is quite variable as observed for all ceramide spectra. The band generally lies between 3700 cm^{-1} and 3100 cm^{-1} with peaks arising at 3280 cm^{-1} and around 4000 cm^{-1} . Those ceramides rich in 2-hydroxy fatty acids such as ceramides derived from cerebroside (e.g. brain ceramide band 2 Fig. 3.6), show stronger hydroxyl absorption as indicated by the peak at 3320 .

The variable intensity and presence of 2, 3 or 4 absorption bands in the 3700 cm^{-1} to 3040 cm^{-1} region is largely due to the many types of hydrogen bonding⁽⁵⁵⁾ that can occur, intermolecularly and intramolecularly, in the ceramide species. The basic nature of the carbonyl oxygen, hydroxyl oxygens and amino nitrogen provides a means of linkage of the hydrogens of the highly polar O-H and N-H groups. The greatest frequency changes with linkage distance occur in the order OH---O, OH---N, NH---O and NH---N. Solvent effects were minimised since a non-polar solvent, CCl_4 , was used; the proportion of intermolecularly bonded dimers and polymers was thus greater than if a more polar solvent such as CHCl_3 was used. The interaction of O-H with the highly basic carbonyl system has been shown to cause the largest frequency shifts, due to the strong CO---OH bond; a lowering of up to 340 cm^{-1} has been observed for some acids and secondary amides. Intramolecular bonding could also occur between the carbonyl and 3 hydroxy groups, and the amino and 1-hydroxy group providing the geometry of the molecule allows this; hydroxy fatty acids would increase the bonding. Interaction

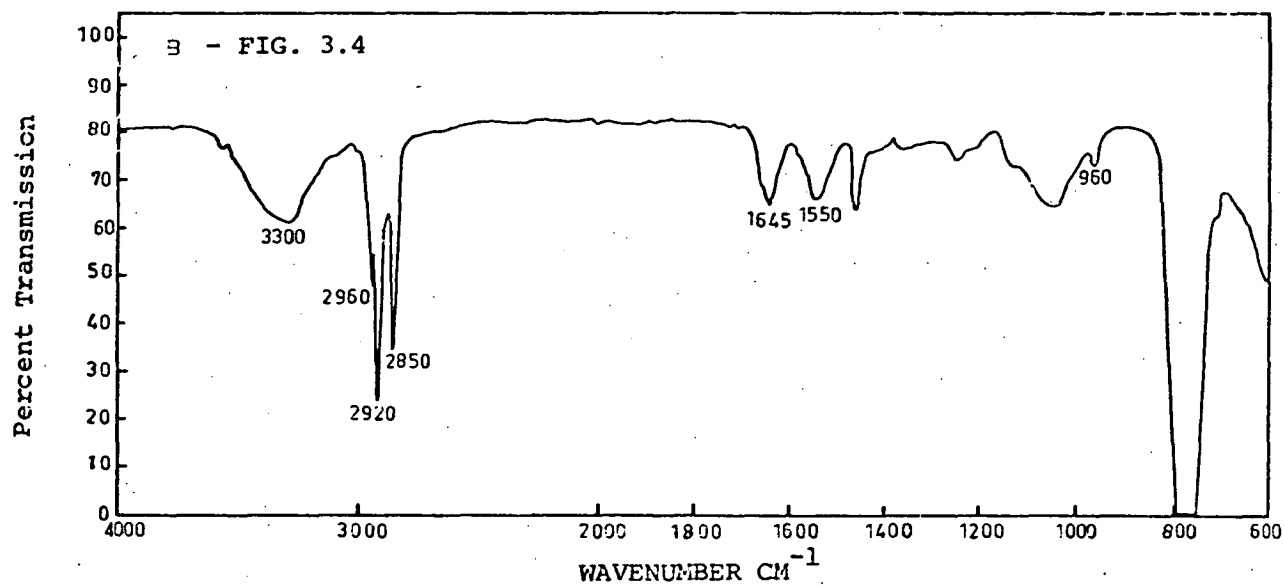
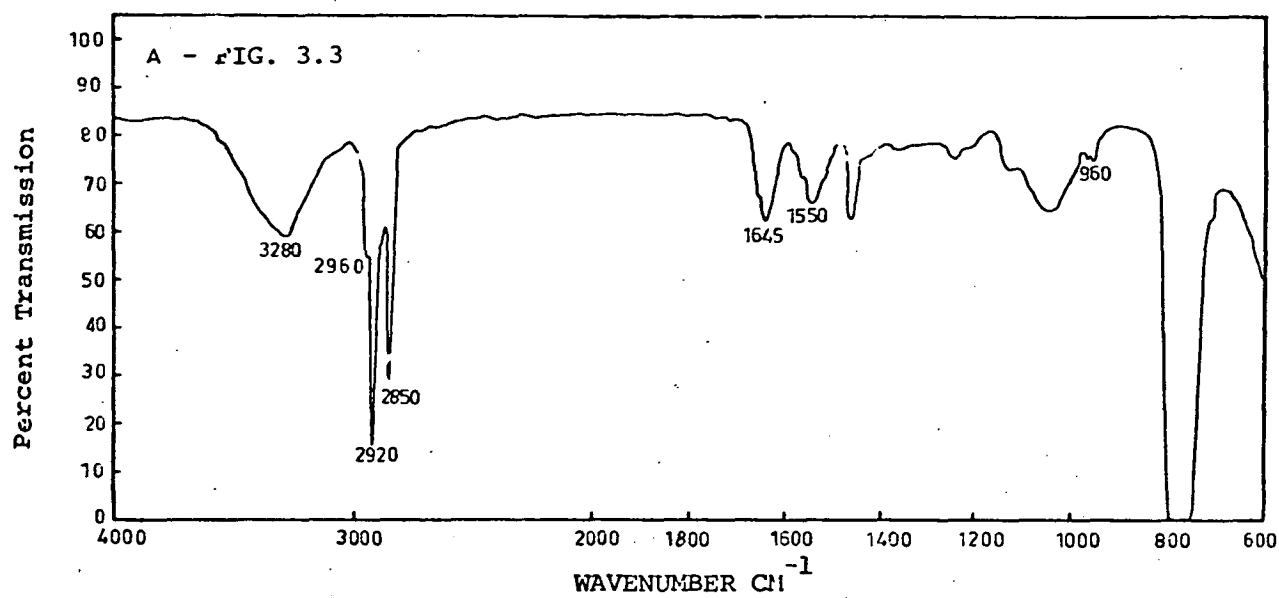
between the polar groups and π -unsaturation centres can also occur, with frequency shifts between 20 cm^{-1} and 100 cm^{-1} depending on the basicity of the π cloud; intramolecular bonding gives an increase in intensity of the O-H stretch.

Amides self-associate very strongly especially in moderate concentration in non-polar solvents, The frequency and intensity varies with concentration towards a lower frequency due to the formation of H-bonded polymers, and also due to Fermi resonance with the overtones of the lower frequency deformation modes in trans secondary amides (i.e. the amide II band). An absorption band at $3060\text{--}3100$ has been observed by Nyquist⁽⁵⁶⁾ for secondary amides in CCl_4 which are highly associated; this had been found to be due to the first overtone of the amide II band (Miyazawa)⁽⁵⁷⁾, which for ceramides (straight-chain alkyl) has the trans configuration. The absorption for trans secondary amides acquires its intensity from Fermi interaction with the bonded N-H stretching vibration. The intensity has been shown to decrease with decreasing association by Nyquist⁽⁵⁶⁾ and Beer et al.⁽⁵⁸⁾ when the amide II band shifts to a lower frequency while the bonded νNH band increases in frequency and decreases in intensity.

The same absorption band (3080 cm^{-1}) is also observed to be present in the spectra of bands 1 of all ceramides synthesised and naturally occurring; it is much reduced in the other bands, of which the amide II vibration frequency is lowered by about $20\text{--}30\text{ cm}^{-1}$ from 1550 cm^{-1} of band 1. The shift of νNH to a lower frequency is not observed however, and the very large overall increase in intensity of $\nu\text{NH-OH}$ for synthetic ceramide band 3 and naturally occurring ceramide band 2 is not fully understood.

Another phenomenon associated with the band near 3100 cm^{-1} for secondary amides has been the persistence of the band in lactams with

INFRA-RED SPECTRA OF NATURALLY-OCCURRING CERAMIDES



A: EX-BOVINE BRAIN CERAMIDES (KOCH-LIGHT)

B: EX-BOVINE MILK CERAMIDES

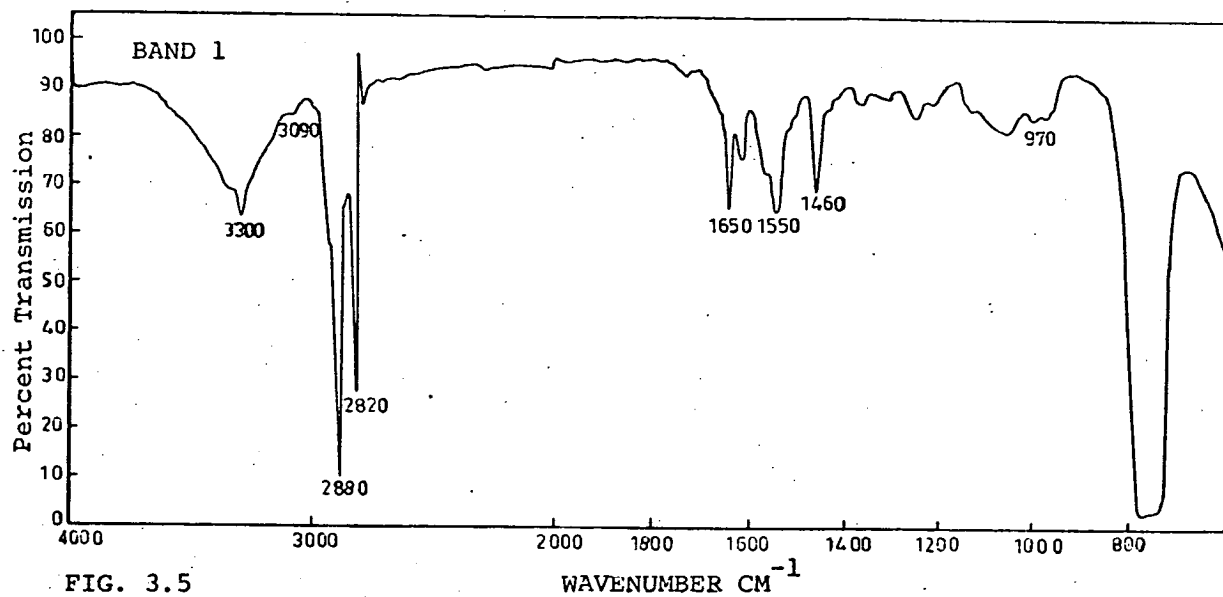


FIG. 3.5

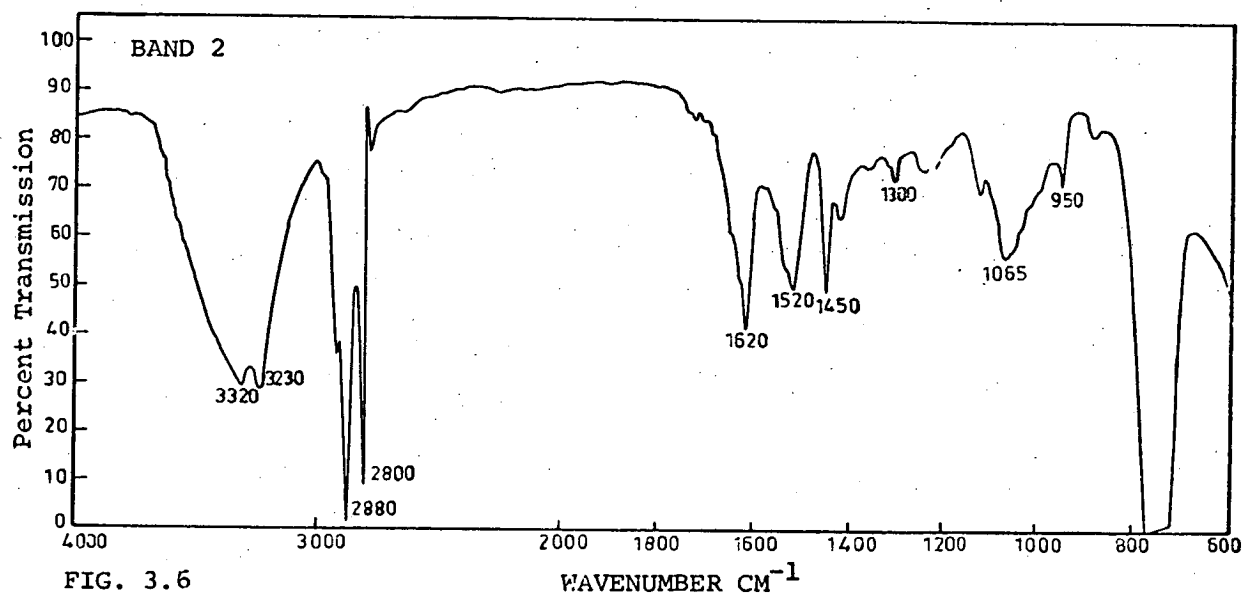


FIG. 3.6

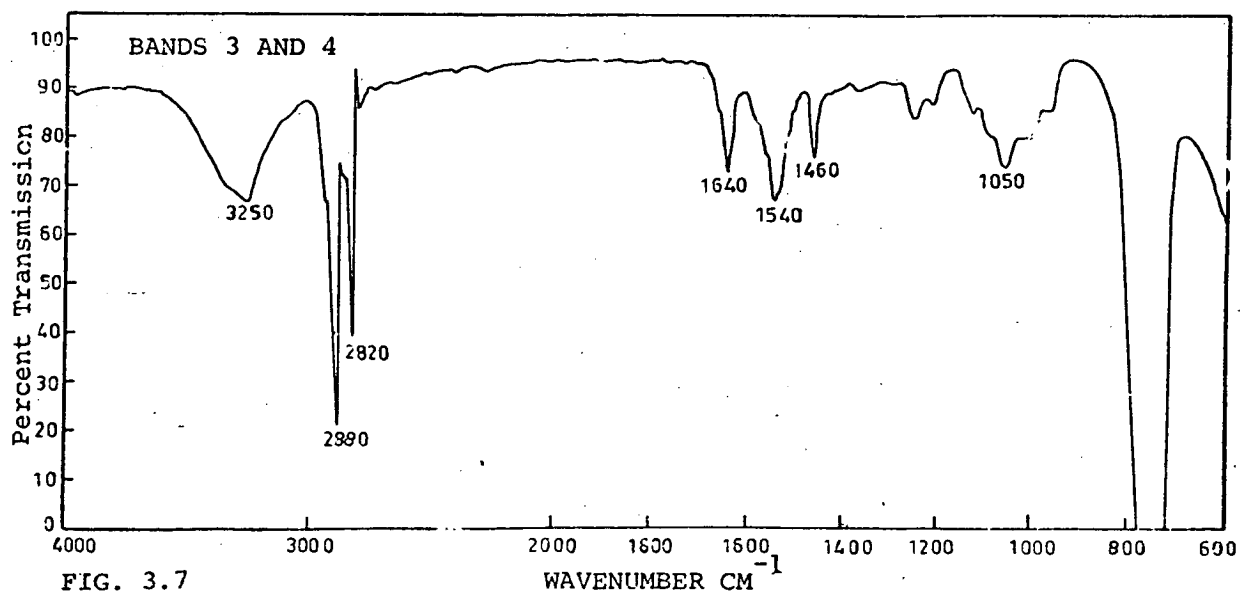


FIG. 3.7

T.L.C. BANDS. 1, 2, & 3, 4 OF EX-BOVINE BRAIN CERAMIDES (KOCH-LIGHT)

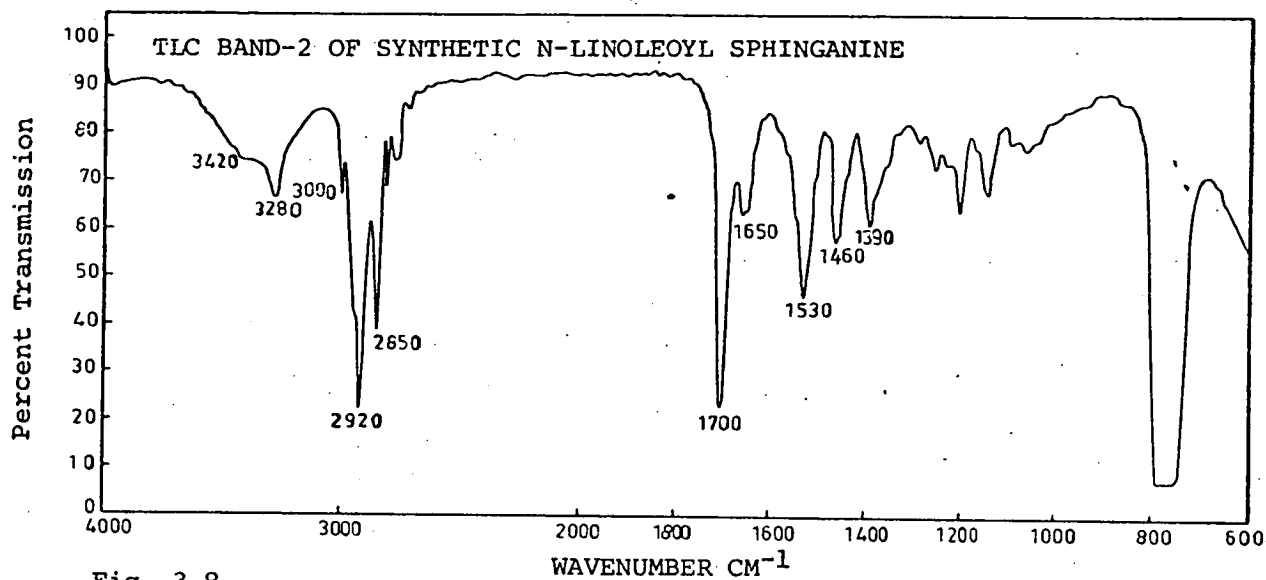


Fig. 3.8

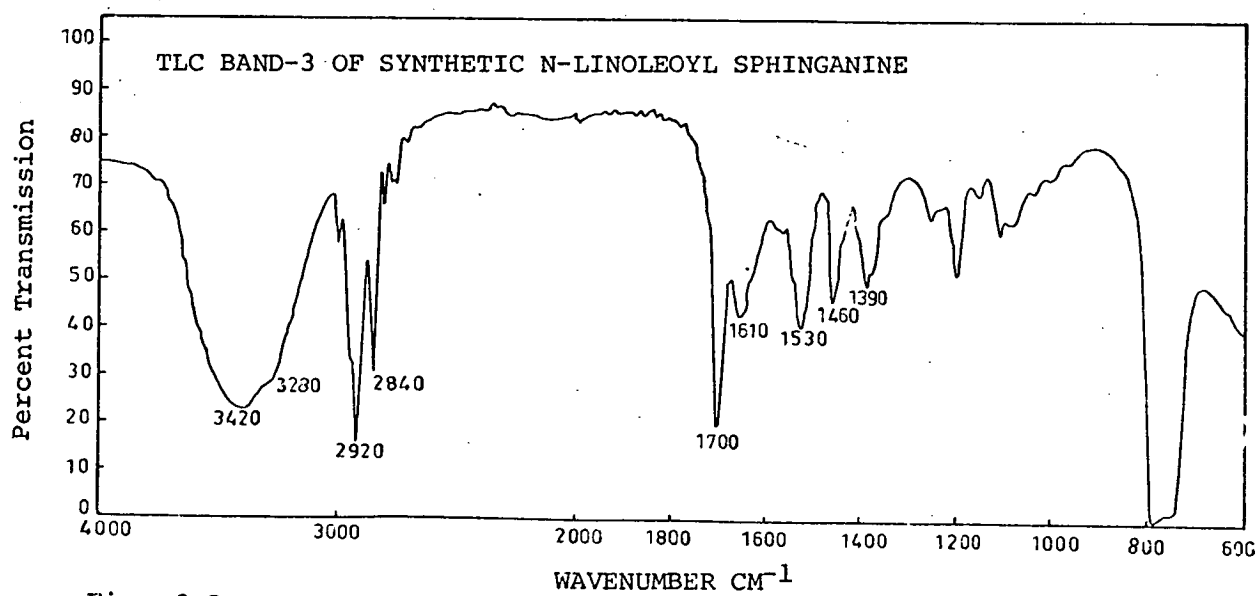
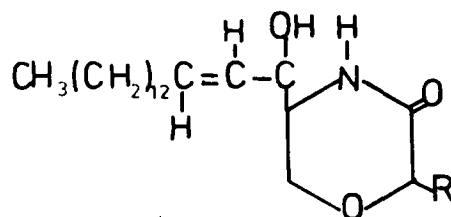


Fig. 3.9

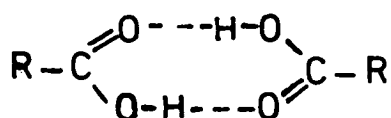
up to 9 members in the ring, of which only the cis form exists as shown by Huisgen et al.⁽⁵⁹⁾ (10- or more membered rings were shown spectrally to resume the trans configuration). The amide II band was found to be absent in the 1550 cm^{-1} region for cis lactams, but Miyazawa⁽⁵⁷⁾ identified it in the 1440 cm^{-1} region and assigned the 3100 cm^{-1} band as a combination NH-in-plane mode and carbonyl absorption. The formation of cyclic amides from ceramides could be a possible explanation for the unidentified ceramide bands observed, in addition to the epoxides and oxazolines previously mentioned. These would have a structure similar to the following:



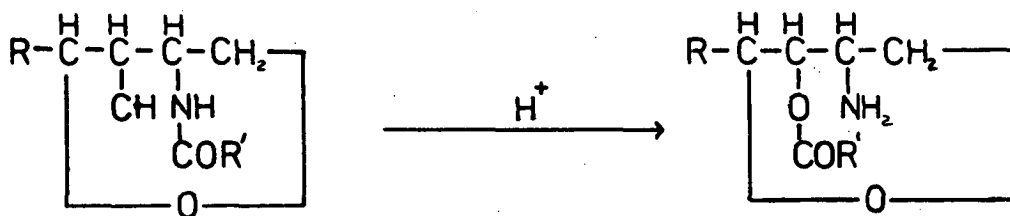
Caprolactam has bands at about 3303 , 3217 , 3088 and 1660 cm^{-1} (59).

A similar group of absorption bands is observed for N-linolecyl-sphinganine band 1, at 3280 , 3200 , 3070 and 1640 cm^{-1} . The NH deformation at 1440 cm^{-1} is not apparent however, so that the lactam may be in small concentration only (the strongest band in caprolactam was at 3217 cm^{-1} , which is weak comparatively here).

The intense absorption bands occurring at 1705 cm^{-1} for band 2 and 3 of the synthetic ceramides (Figs. 3.8, 3.9) are undefined as yet, and could be due to a number of possibilities. Some carboxylic acid could be associated with the ceramide (though washed with sodium bicarbonate). The 1705 cm^{-1} band would then correspond to the C=O stretch of the acid dimer, and a number of small bands at 2770 , 2710 and 2660 cm^{-1} on the broad diffuse $3000\text{--}2500\text{ cm}^{-1}$ OH stretch would be due to OH absorption of the intermolecularly hydrogen bonded dimer.



Another possibility could be the migration of the acyl group from the N position to the neighbouring cis hydroxyl group by the action of dilute acid, so producing an O-acyl ester. Such an acyl migration has been observed in anhydro-phytosphingolipids⁽⁷¹⁾, and infrared spectroscopy showed the appearance of the ester band while the amide band disappeared



Anhydro-compounds similar to the above compounds would be unlikely to form with sphinganine ceramides but could with sphing-4-enine ceramides, in the event of the formation of 1,2 epoxides, followed by cleavage of the epoxide ring with dilute acid to form 1,2 glycols.

Mass Spectra of Synthetic Ceramides

TLC bands 1,2, and 3 of the synthetic ceramides N-oleoyl sphinganine and N-linoleoyl sphinganine, and N-hexanoyl sphinganine were subjected to mass spectrometric analysis as their trimethylsilyl-ethers according to the method of Samuelsson and Samuelsson⁽⁶⁰⁾. The TMS derivatives were prepared by treating the lipid (dry, ca. 1 mg) with 100 μl of anhydrous pyridine, 20 μl of BSTFA, and 10 μl of TMCS at room temperature for an hour. The solvent was evaporated to dryness in a stream of nitrogen, dried in an evacuated dessicator and dissolved in ethyl ether (peroxides removed by acidified ferrous sulphate, dried and distilled) to be analysed directly.

The mass spectra of the pure N-linoleoylsphinganine band 1 and N-hexanoylsphinganine are represented by Figs. 3.11 and 3.15, respectively. The mass spectrum of N-oleoylsphinganine was similar to that already reported⁽⁶⁰⁾, differing from the spectrum of N-linoleoylsphinganine only in the fatty acid fragments, which were two units higher. The spectrum of N-hexanoylsphinganine may be analysed as follows. The molecular weight is indicated by ions at m/e 528 (M-15), m/e 453 (M-90), and m/e 440 (M-103) by the loss of a methyl group, trimethylsilanol and the terminal $-\text{CH}_2-\text{O}-\text{Si}(\text{CH}_3)_3$, respectively. The molecular ion M^+ thus occurs at m/e 543. Two major ions present involve cleavage between C2 and C3; charge retention on the sphinganine fragment results in an ion at m/e 313 (M-d), and the ion at m/e 230 (M-a) represents the same cleavage but with charge retention on the other part of the molecule. Other long-chain base fragments just observable are those occurring at m/e 428 (M-(b+1)) and m/e 338 (M-(b+1)-90), produced by the loss of the hexanoyl amide, and hexanoyl amide plus trimethylsilanol, respectively. An ion that is characteristic of all sphinganine ceramides is produced by loss of the acyl amide plus $\text{CH}_3(\text{CH}_2)_{12}$ of the sphinganine moiety; this ion occurs at m/e 217 (M-(b+1)-c). The long-chain base fragments given above are the same for all C18:0 sphinganine ceramides (i.e. M-d=313, M-(b+1)=428, and M-(b+1)-90=338 as determined by Samuelsson; the ions occur two mass units lower for C18:1 sphingosine ceramides.

Several fatty acid fragments are formed as a result of cleavage between C2 and C3 of the sphinganine molecule. Prominent ions occur at (M-a-89) and (M-a+73) after cleavage, and these have been assigned the elimination of the trimethylsilyloxy radical at C1, and transfer of the C3 trimethylsilyl group to the nitrogen, respectively; for N-hexanoylsphinganine, the ions occur at m/e 141 and m/e 303, and for N linoleoylsphinganine the ions occur at m/e 305 and m/e 467. The ion formed by

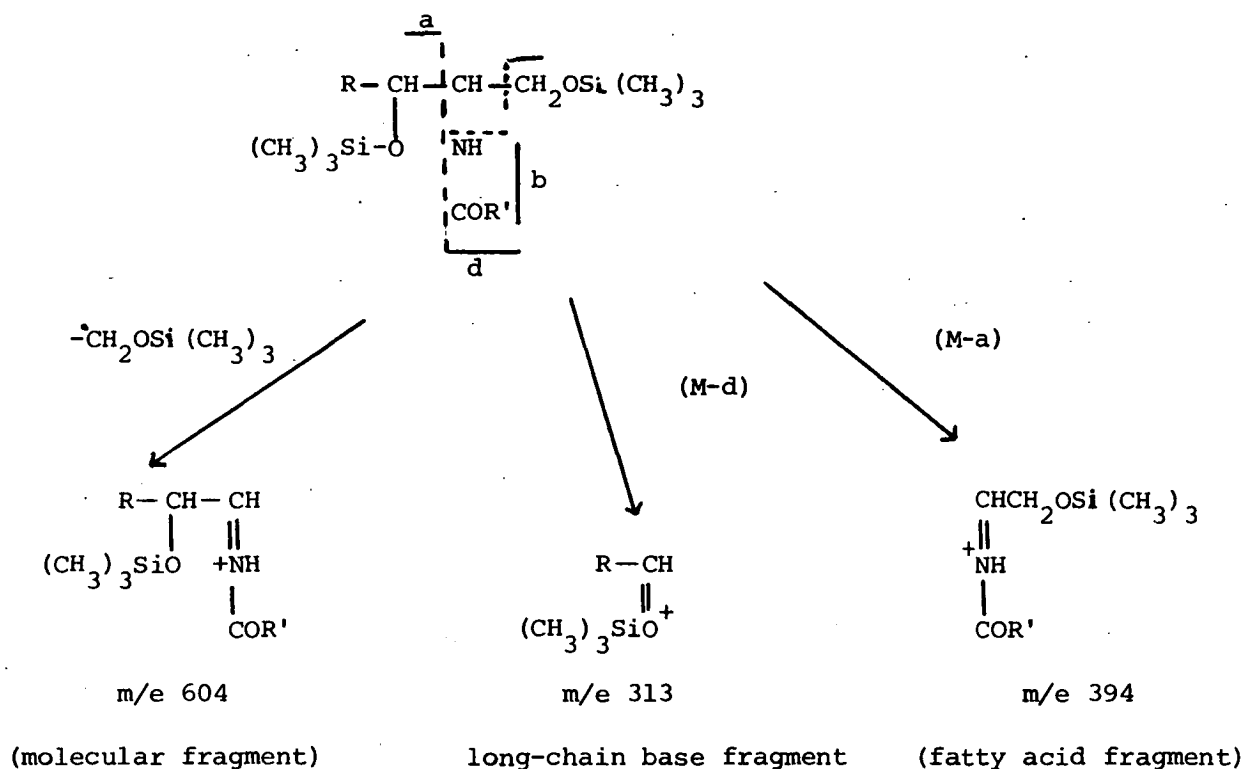
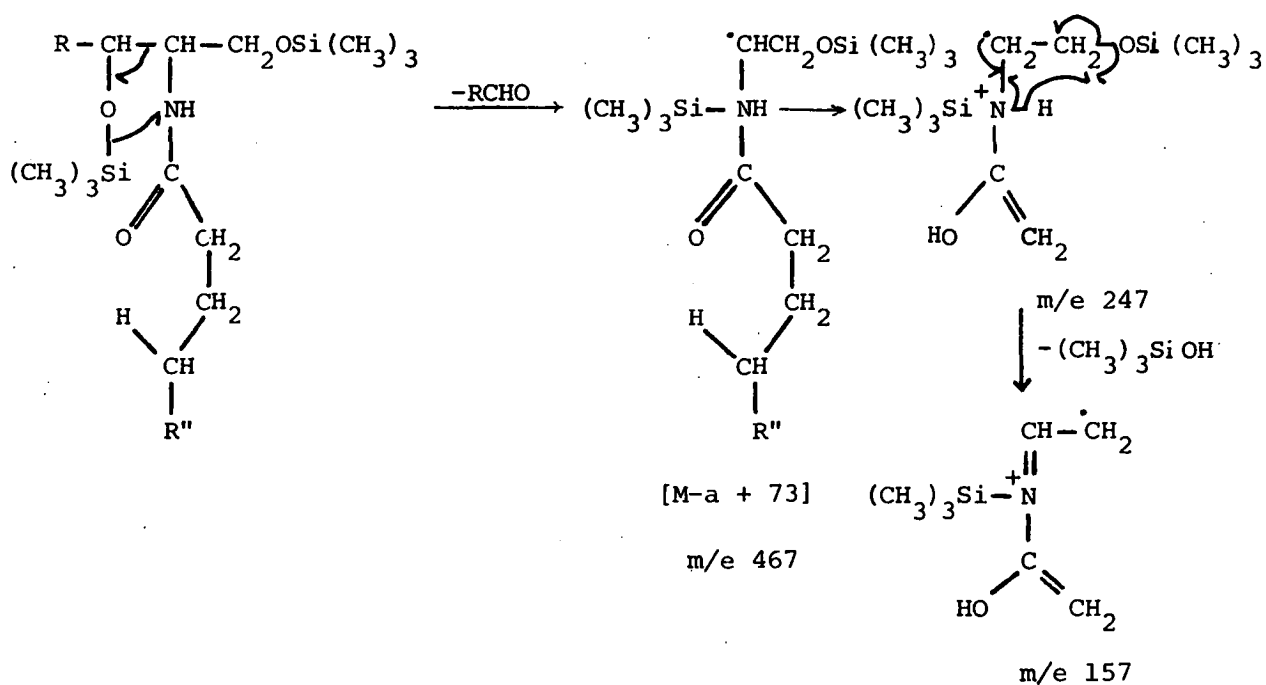
C2-C3 cleavage with transfer of the trimethylsilyl radical ($m-a+73$) undergoes a McLafferty type rearrangement, i.e. β -cleavage of the acyl group with transfer of the γ -hydrogen to the carbonyl oxygen. For ceramides with non-hydroxy acids, Samuelsson (60,61) and Hammarstrom (62) have shown by deuterium substitution on the fatty acyl moiety that only three deuterium atoms are retained after the McLafferty rearrangement. The ion occurs at m/e 247 ($M-1-73$) for the hydrogenated species, and m/e 250 for the deuterated species; the additional elimination of trimethylsilanol gives rise to an ion at m/e 157. These ions are observed to be present in the mass spectra shown.

Some of the major fragmentation modes are represented on the following page for N-linoleoylsphinganine. The structure of this ceramide is also represented by its nuclear magnetic resonance spectrum (Fig.3.12). The four olefinic protons resonate as a sextet centred at 4.67 ppm. while the peak centred at 7.25 ppm. integrates for the two methylene protons between the olefinic double bonds. The methylene protons adjacent to the double bonds and to the carbonyl oxygen are observed to resonate between 7.7 and 8.1 ppm; the methylene and methine protons adjacent to the double bonds and to the carbonyl oxygen are observed to resonate between 7.7 and 8.1 ppm; the methylene and methine protons adjacent to the hydroxyl and amino groups are thus assigned to the peaks centred at 6.22 and 6.05 ppm. The bulk of the protons, methylene and methyl, resonate between 8.6 and 8.9 ppm. (the peak at 9.0 - 9.2 ppm. arises when compounds are extracted from silica gel plates from N.M.R. spectral analysis). No portion of the above peaks collapsed on treating the ceramide with D_2O ; OH and NH protons were not detected, and probably occurred as broad diffuse bands over the spectrum.

Some evidence for the structure of the ceramide bands 2 and 3 of the synthetic ceramides can be derived from their mass spectra.

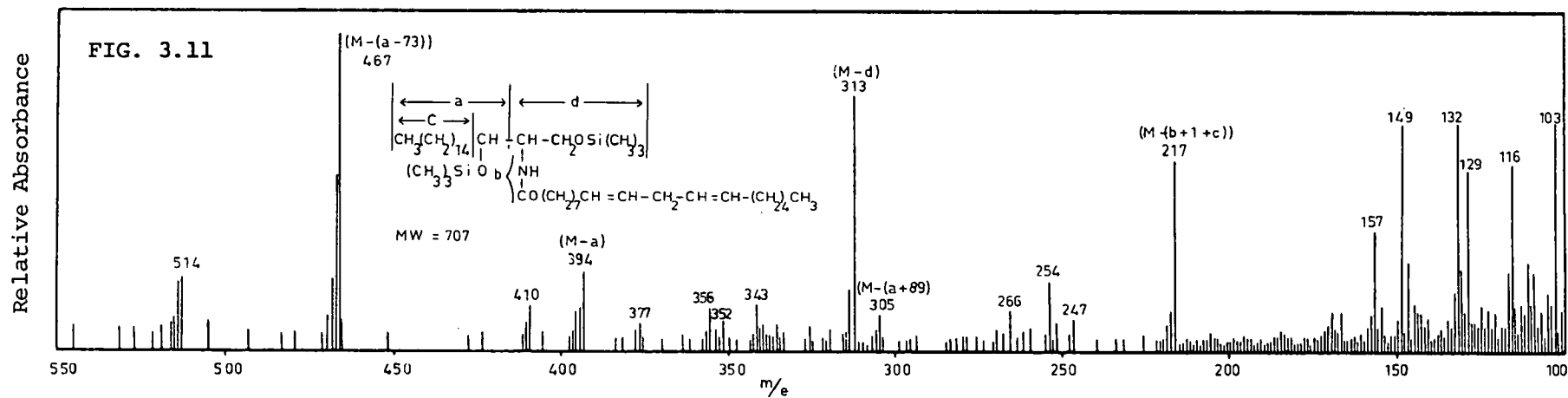
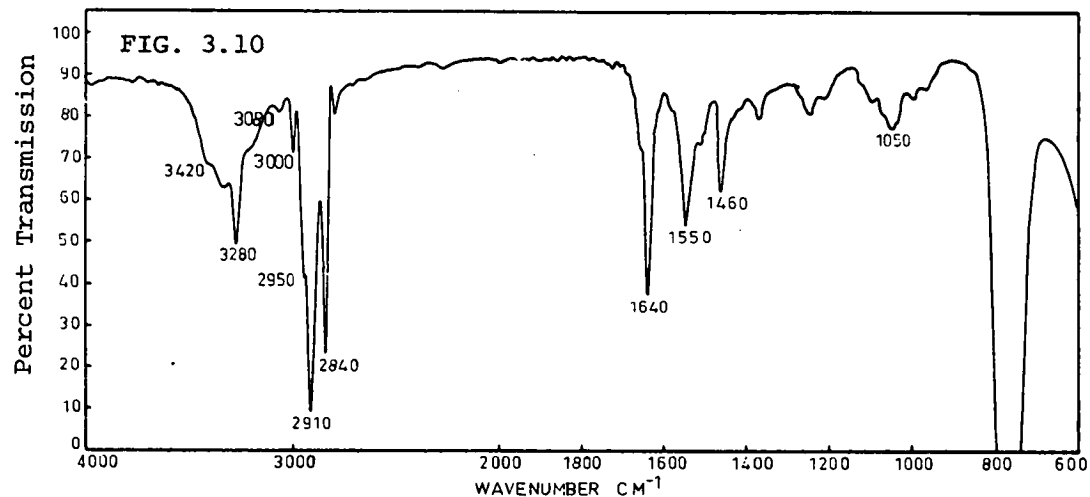
Mass Spectral Fragmentations that Characterize Ceramides

e.g. N-linoleoyl-DL-sphinganine (M.W. = 707)

Proposed formation of (M-a + 73) - Samuelsson^(60,61)

The major ions that characterize ceramides, and that were prominent also in the spectra of bands 2 and 3 were found by comparison with the spectrum of band 1 to be (M-d), (M-(b+1)), (M-(b+1)-90), and (M-(b+1)-c); these are the fragments corresponding to the long-chain base, and the latter to a fragment observed for all sphinganine derivatives. Also present in all spectra was the ion occurring at m/e 247 which occurs as a result of β cleavage with a rearrangement of the fatty acid fragment, i.e. (M-a+73); the ion at m/e 157 was also present (loss of trimethylsilanol). However, the parent ion (M-a+73), a major peak in the spectrum of band 1, was absent in bands 2 and 3, as were other fatty acid fragments (M-a) and (M-a-89). These observations suggest that the sphinganine base is straight-chained past C2, and that the fatty acid moiety may have a different molecular structure; the terminal carbon C1 of the long chain base must have the trimethylsiloxy radical due to the ion at m/e 247. The presence of unstable very intense ions at m/e 435 and m/e 364 in bands 2 and 3, absent in band 1 for N-linoleoylsphinganine (m/e 437 and m/e 366 for N-oleoylsphinganine) will possibly give some indication of their structures in the future.

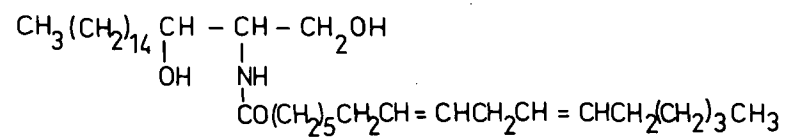
TLC BAND-1 OF SYNTHETIC N-LINOLEOYL SPHINGANINE



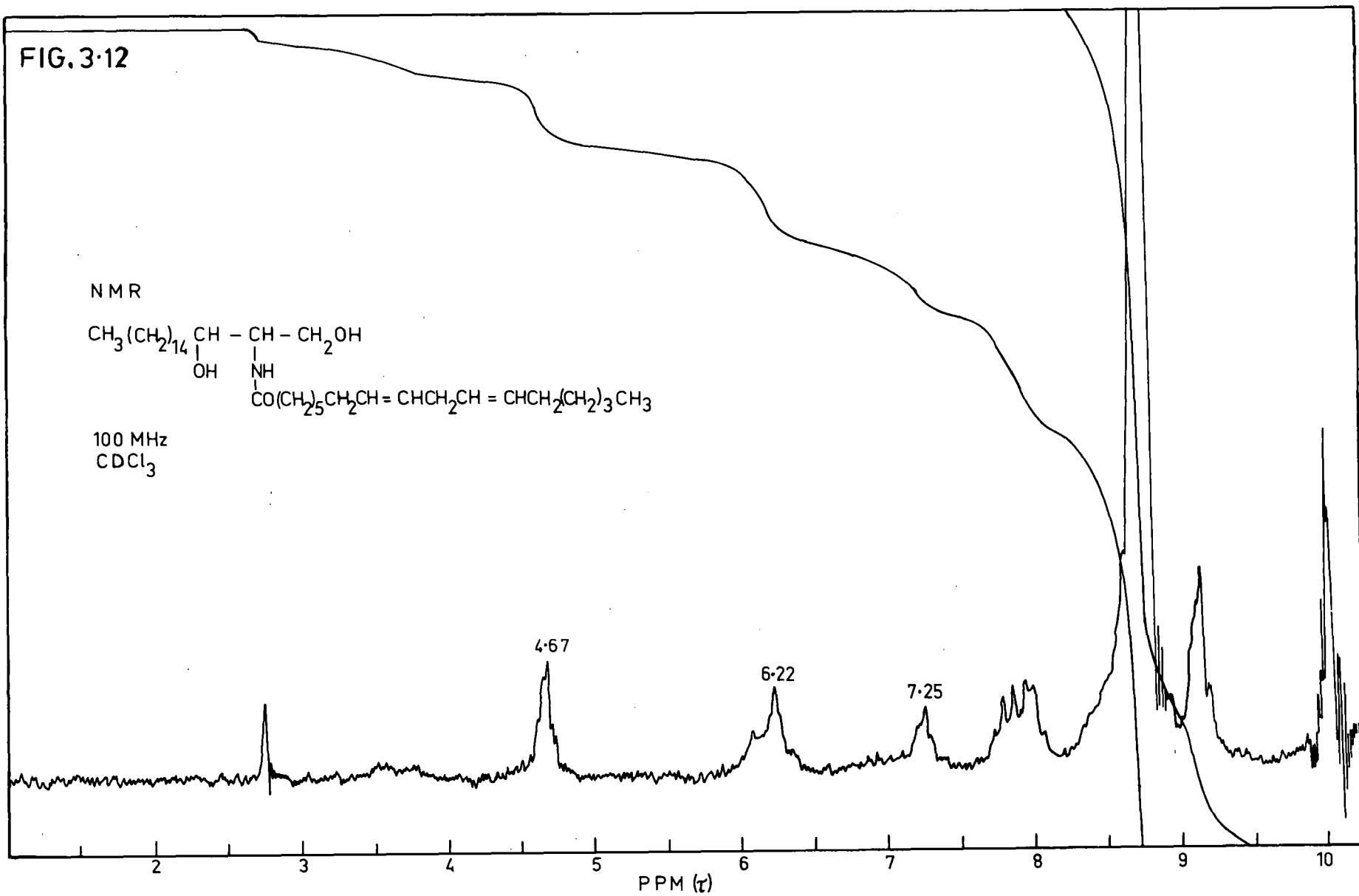
PARTIAL MASS SPECTRUM OF 1,3-DI-O-TRIMETHYLSILYL-N-LINOLEOYL SPHINGANINE

FIG. 3.12

NMR



100 MHz
CDCl₃



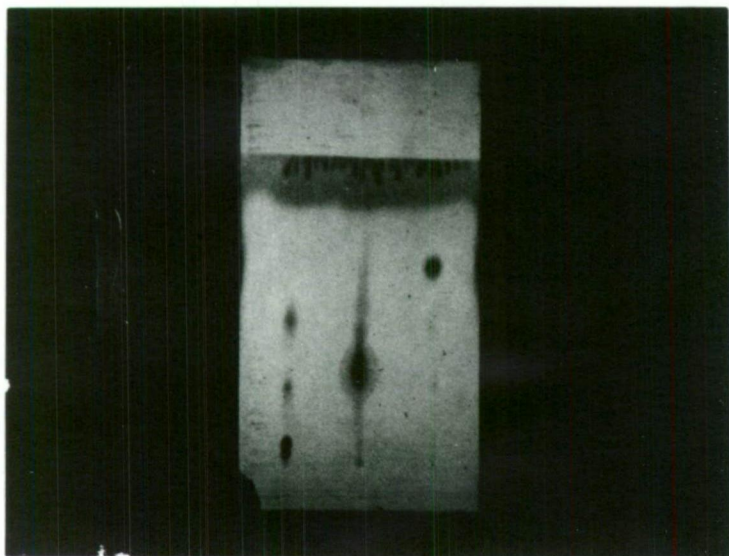


FIG. 3.13

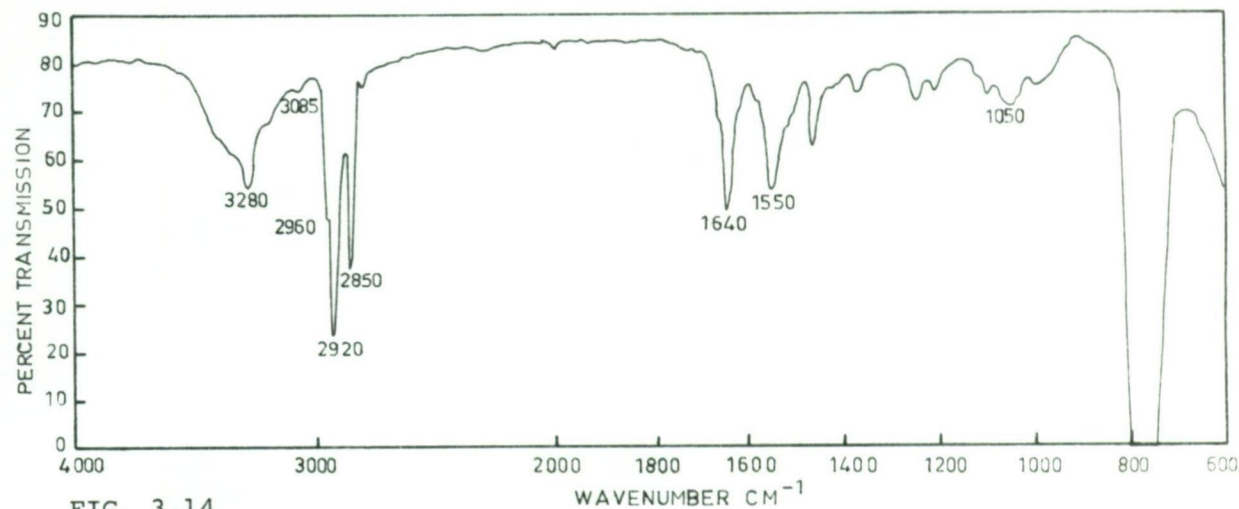


FIG. 3.14

TLC AND INFRA-RED SPECTRUM OF N-HEXANOYL-SPHINGANINE

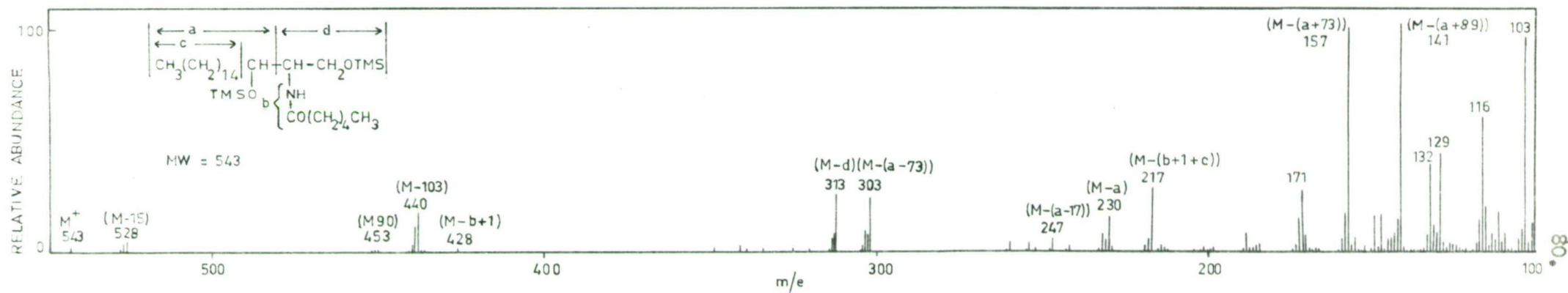


FIG. 3.15

MASS SPECTRUM OF 1,3-DI-O-TRIMETHYLSILYL-N-HEXANOYL-SPHINGANINE

CONCLUSIONS

Infrared and mass spectroscopic methods will undoubtedly be the most powerful methods for the structural characterization of ceramides and their derivatives. This has been proved by experts in the field such as Samuelsson, and Hammarstrom, who have used combined gas-liquid chromatography - mass spectrometry for the analysis of synthetic ceramides^(60,61) containing phytosphingosine⁽⁶²⁾ or 2-hydroxy acids,⁽⁶³⁾ and naturally occurring ceramides⁽⁶¹⁾ in human platelets⁽⁶⁴⁾ or derived from human plasma sphingomyelins⁽⁶⁵⁾ or brain cerebroside.⁽⁶⁶⁾ The long chain bases derived from sphingolipids have also been analysed by combined GLC-MS of the aldehydes obtained on periodate oxidation by Sweeley and Moscatelli,⁽²⁸⁾ Morrison^(19,20) and Panganamala et al.⁽⁶⁷⁾ who prepared the 1,3 dioxalanes from the derived aldehydes. Karlsson⁽⁶⁸⁾ has also prepared the dinitrophenyl derivatives of the long chain bases and analysed them by GLC-MS after separation by TLC.

The analysis of long chain bases in milk ceramides may be carried out by the above methods, perhaps using a fraction collector to isolate the long chain bases before subjecting them to mass spectroscopy. The positions of the double bonds could be determined by methods previously used. Politio et al.⁽⁶⁹⁾ have analysed the N-acetyl-O-trimethylsilyl derivatives of sphingolipid bases by GLC-MS after oxmium tetroxide oxidation of the double bonds. The location of the double bonds was evidence by mass spectral fragmentations at positions of vicinal trimethylsiloxy groups. Hayashi and Matsubara⁽⁷⁰⁾ have also used periodate-permanganate oxidation to determine the structure of sphinga-4,8-dienine. These methods could also be applied to the methyl and acetoxy methyl esters of the fatty acids in milk ceramides, to provide conclusive evidence of the chain length, double bond positions and hydroxyl positions (primarily 2-hydroxy fatty acids have been found to exist in animal lipids).

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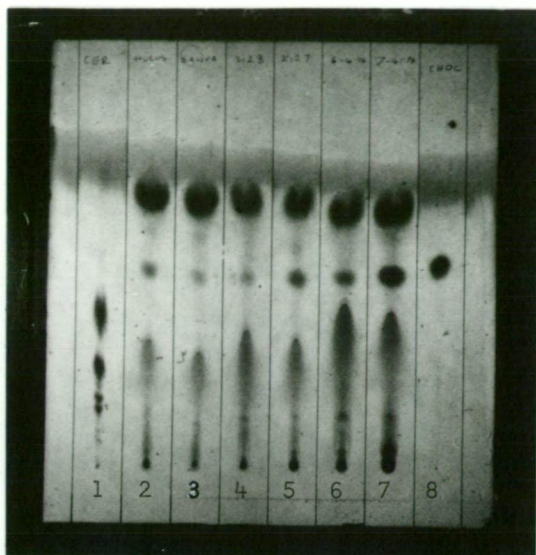
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APPENDIX 1.

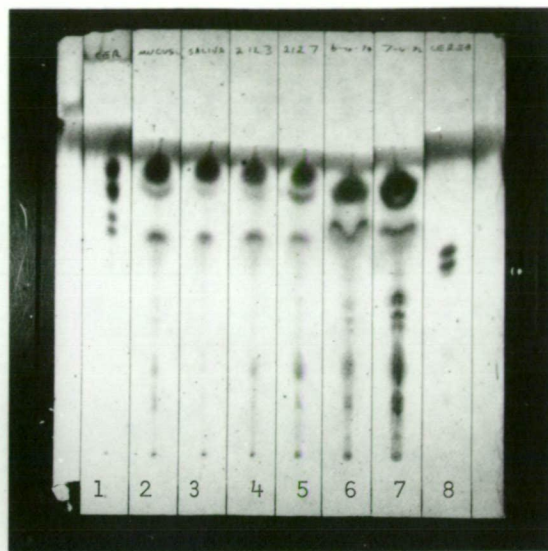
Free ceramide is observed to be present to a very small extent in all animal tissues. However, in the excretions or tissues of sick organisms it is observed to be present in a much greater quantity⁽⁴³⁾. The lipids from two-excretion-types were tested by TLC, sputum (obtained via Dr. Parsons, Hobart, Tas.) from asthamatic patients, and ambergris, the excretion from sick whales. A red-backed spider was also tested.

The level of ceramide is observed to increase in about 20 cases examined of people who suffer from asthma, by TLC of the extracted lipids shown on photographs 9-12. The ceramide occurs at about R_f 0.45, similar in position to Koch-Light ceramide band 2; a small band occurs just above this in some cases, probably derived from sphingomyelin in a similar manner as was found in bad milk. Cholesterol is present to a relatively large extent in most cases; cerebrosides are observed in high concentration when ceramide and cholesterol concentrations are high.

A ceramide spot at R_f 0.45 was also observed to be present in ambergris, and in the red-back spider. The ambergris lipids contained mostly cholesterol and its esters, with only a little triglyceride. A compound similar in appearance but more greasy than ambergris was also tested, and found to contain very little of a spot around R_f 0.4. It was mostly triglycerides, as shown by TLC. By soxhlet extraction it was found to be 95% soluble in chloroform, the residue another 1% soluble in methanol to form a pale yellow solution, and 4% insoluble in organic solvents. The yellow residue did give rise to a faint spot in the ceramide region on TLC; irradiation of the residue with U.V. light for 8 hours did not affect this TLC spot.

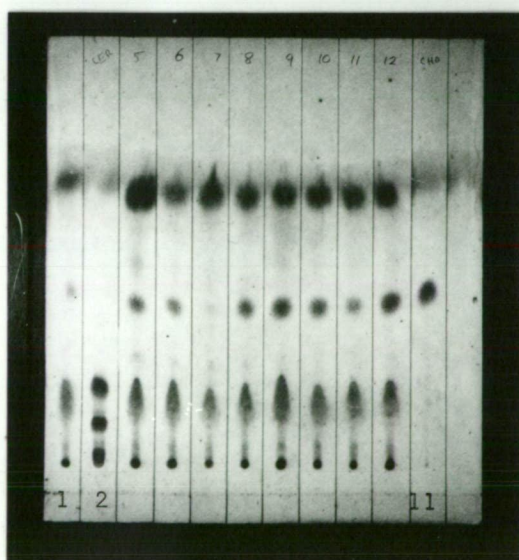


9

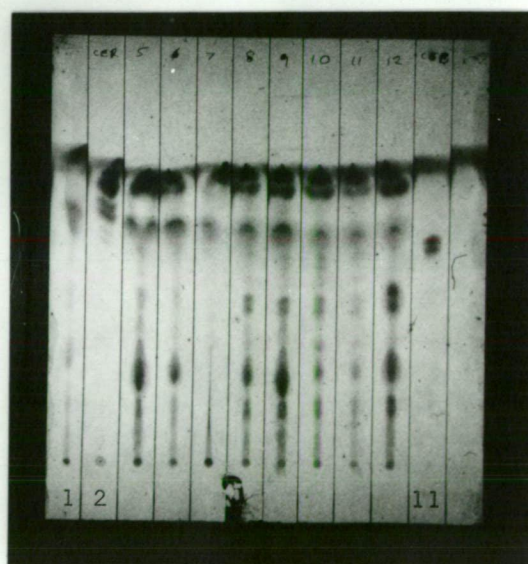


10

(1) Brain ceramides; (2) Control mucus sample; (3) Control saliva sample; (4) to (7) Asthmatic sputums; (8) cholesterol (p.9) or cerebrosides (p.10).



11



12

Columns marked 5 to 12 represent lipids from sputum samples from asthmatic patients. Column 1 is a control; col. 2 = ceramides; col. 11 = cholesterol (p.11) or cerebrosides (p.12).

Plates 9,11 - sol. A.

Plates 10,12 - sol. B.